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THE ROLE OF NITRIC OXIDE IN NICOTINIC RECEPTOR INDUCED MYOPATHY

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A Thesis submitted to the Faculty of Graduate Studies and Research
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for the degree of Masters of Science

This thesis is dedicated to my Parents, Fatima Krisht and David El-Dada. Whatever I do and to wherever the path of life may lead me, You will always be in my heart ....
Your wisdom and words will light up my way.....

When Things Go Wrong, As They
Sometimes Will,
When The Road You're Trudging
Seems All Uphill,
When The Funds Are Low And The Debts
Are High, And You Want To Smile But You Have To
Sigh,
When Care Is Pressing You Down A Bit
Rest if You Must, But Don't you Quit

Anonymous

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PREFACE

A note on the format of this thesis:

In accordance with the regulation of the Faculty of Graduate Studies and Research, the candidate has the option of including as part of the thesis the text of original papers already published by learned journals, or those submitted or suitable for submission to learned journals. The text relating to this is as follows:

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If this option is chosen, **connecting text that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements explained of the **" Guidelines for Thesis Preparation". The Thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers."

This thesis is divided into two experimental chapters that will be referred to as:

Chapter 2: Involvement of nitric oxide in nicotinic receptor mediated myopathy. (accepted for publication in a modified form in *Journal of Pharmacology and Experimental Therapeutics*, **1997**)

Chapter 3: The effect of nicotine on nitric oxide synthase activity in neonatal muscle cells in culture.

TABLE OF CONTENTS

TABLE INDEX

TABLE

FIGURE INDEX

FIGURE

LIST OF ABBREVIATIONS

α -BGT	Alpha-bungarotoxin
AMPA	Alpha-amino-3-hydroxy-5-methyl-isoxazole
BH_4	Tetrahydrobiobterin
BSA	Bovine serum albumin
BMD	Becker's muscular dystrophy
CNS	Central nervous system
Cytosar	Cytosine arabinoside
DAG	Dystrophin-associated glycoproteins
DAP	Dystrophin-associated proteins
DMD	Duchenne's Muscular Dystrophy
DMEM	Dulbecco's modified Eagles medium
D-NAME	D-nitroarginine methyl ester
EDTA	Etylenediaminetetraacetic acid
EDRF	Endothelium-derived relaxing factor
EGTA	1,2 Di-2-aminoethoxyathane-N, N, N',N'-tetraacetic acid
FAD	Flavin adenine dinucleotide
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanylate cyclase
GSNO	S-nitroso glutathione
h	Hour(s)
IGF-I	Insulin like growth factor-I
IRE-BP	Iron responsive element binding protein
IRF	Iron responsive factor
L-NAME	L-nitroarginine methyl ester
LTP	Long term potentiation
MCK	Muscle creatinine phosphokinase
min	Minute(s)

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
nAChR	Nicotinic acetylcholine receptor
NANC	Non adrenergic non cholinergic neurotransmission
NIO	L- ^{5}N-iminoethyl ornithine
NMJ	Neuromuscular junction
NO	Nitric oxide
NOS	Nitric oxide synthase
PARS	Poly(ADP-ribose) synthetase
$O_2^{\cdot}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite anion
ROI	Reactive oxygen intermediates
S.E.M.	Standard error of the mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SERCA2	Sarcoplasmic reticulum Calcium ATPase
SR	Sarcoplasmic reticulum

ABSTRACT

Previous studies have shown that inhibition of acetylcholinesterase, which increases acetylcholine levels at the neuromuscular junction (NMJ) produces muscle necrosis. *In vitro* studies have also demonstrated that the application of cholinergic agonists results in muscle damage which was prevented by calcium chelators or nicotinic receptor blockade. Nitric oxide (NO), a free radical which mediates intra- and intercellular processes, has been shown to play a role in NMDA glutamate receptor mediated excitotoxicity. Recent studies have demonstrated the presence of the calcium-calmodulin dependent isoforms of NO synthase (NOS) in skeletal muscle. These results may suggest a possible role for NO in the agonist-induced myopathy at the NMJ. The present experiments were carried out to test the hypothesis that NO is involved in nicotinic receptor mediated muscle cell degeneration. As a model to study nicotinic receptor mediated myopathy, neonatal skeletal muscle cultures prepared from one day old Sprague-Dawley pups were used. Skeletal muscle cultures were exposed to different concentrations of nicotine. The results demonstrate a significant dose dependent decrease in the number of muscle branch points with increasing nicotine concentrations. The degenerative effect(s) of nicotine were prevented by preincubating the muscle cultures with d-tubocurarine, a nicotinic receptor blocker, suggesting that the effects of nicotine are receptor mediated. Experiments were then done to assess an involvement of the NO generating system in the nicotine induced degeneration. NO synthase (NOS) inhibitors prevented/inhibited the nicotine induced degenerative effects on myotube size and branching, while exposure of the cells to sodium nitroprusside (SNP), an agent which releases NO spontaneously, resulted in myotube degeneration. As another approach to determine an involvement of NO in the

myopathy, the effect of nicotine on NOS activity was determined. NOS activity increased in a time and tissue dependent manner in neonatal rat skeletal muscle cultures. NOS activity was then determined in the absence or presence of nicotine. The results indicated an increase in NOS activity in the muscle cultures upon exposure to nicotine. These experiments suggest a possible role for NO as an intracellular messenger mediating the myopathic effects of nicotine in rat skeletal muscle cells.

, un agent producteur du NO, provoque de dégénérescence des myotubes. Comme autre approche pour déterminer l'implication du NO dans les myopathies provoquis par la nicotine, l'activité de la synthase á été déterminée. Cette activité augmente avec le temps d'incubation et selon les concentrations de tissues utilisés. Des expériences ont alors été faites pour déterminer l'activité de la NO synthase en absence ou en présence de nicotine. Les resultats indiquent une augmentation de l'activité de la NO synthase aprés exposition des cultures musculaires à la nicotine. Ces expériences suggérent un rôle du NO dans les effets de la nicotine sur les myopathies.

SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this thesis, rat skeletal muscle cells in culture were used to study the role of NO in nicotinic receptor-induced myopathy. Studies using neonatal skeletal muscle cultures prepared from 1-2 day old Sprague-Dawley rats were used as a model to address this form of agonist-induced myopathy at the neuromuscular junction. The novel findings of this thesis are summarized below.

1. **a)** Nicotine exposure was found to cause a decline in myotube branch points of neonatal rat skeletal muscle cultures. The effect was dose-dependent with increasing concentrations of nicotine.

b) The degenerative effects of nicotine were prevented by pretreatment of muscle cultures with d-tubocurarine, a nicotinic receptor antagonist. This suggests that the degenerative effects of nicotine are receptor mediated.

c) Both, the irreversible NOS inhibitor L-N5 (iminoethyl) ornithine (L-NIO), and L-nitroarginine methyl ester (L-NAME), a reversible NOS inhibitor, protected the skeletal muscle cells from the myopathic effects of nicotine. This suggests a role for NO in the agonist-induced myopathy at the neuromuscular junction.

d) Exposure of muscle cultures to sodium nitroprusside (SNP), a NO donor, resulted in a dose-dependent decrease in myotube branch points with increasing SNP

concentrations. This suggests that SNP mimics the degenerative effects of nicotine.

These results are of significance since they suggest that NO mediates nicotinic receptor-induced myopathy in rat skeletal muscle cultures.

2. a) NOS activity was detectable in the intact rat skeletal muscle cells in culture. The activity of the enzyme increased with increasing tissue concentration.

   b) Nicotine exposure results in an increase in NOS activity in rat skeletal muscle cultures. These findings suggest that the myopathic effects of nicotine are mediated via an increase in NO levels.

These results provide further evidence that NO is a mediator of nicotinic receptor-induced muscle cell degeneration in rat skeletal muscle cells in culture.

1.0. INTRODUCTION

1.1. Skeletal Muscle: Diseases and Regeneration

Muscular dystrophies are a group of diseases that primarily affect skeletal muscle and are characterized by progressive muscle wasting and weakness. Although these diseases have been clinically recognized for a long time, only recently have genetic defects in a number of muscular dystrophies been identified. However, biochemical mechanisms involved in muscle degeneration and regeneration have not been fully characterized.

Muscle fibers are large syncytial cells that contain hundreds of nuclei normally positioned under the plasma membrane. These fibers are in close contact with mononucleated myogenic cells located between the basal lamina and the plasma membrane of the muscle fiber, these satellite cells mediate muscle regeneration. Following injury to a muscle fiber, the satellite cells transform into activated myoblasts, proliferate and fuse into primitive multinucleated myotubes, which then differentiate into mature muscle fibers (Bischoff, 1994).

The NMJ is the most well-characterized peripheral synapse between nerve and muscle. NMJ consists of three highly specialized components: a presynaptic nerve terminal, a postsynaptic muscle cell, and an intervening synaptic cleft which is occupied by a basal lamina.

1.2. The Postsynaptic Apparatus

1.2.1. Nicotinic Acetylcholine Receptors (nAChRs)

nAChRs are well-characterized members of a supergene family of ligand-gated channels that include glycine receptors, $GABA_A$ receptors and 5-HT_3 receptors (Ullian and Sargent, 1995). AChRs

are subdivided into two groups based on their anatomical localization and distribution. Muscle nAChRs are pentameric membrane proteins containing four different kinds of subunits and are postsynaptically concentrated at densities up to 10,000 molecules/μm^2 (Bevan and Steinbach, 1977). Skeletal muscle nAChRs represent an abundant class of cation-selective, ligand-gated ion channels that mediate fast synaptic transmission to the muscle fiber (Corriveau et al., 1995). Mammalian muscle AChR exists in two developmentally regulated isoforms (Hall and Sanes, 1993). In the embryonic muscle, nAChRs are distributed throughout the plasma membrane at a relatively low density with the different subunits having a stoichiometry of [$\alpha_2\beta\gamma\delta$]. However, after the muscle becomes innervated, the γ subunit gene ceases to be transcribed and is replaced by the AChR (ϵ) gene product which also possesses different single-channel properties from the γ-containing embryonic AChR. nAChRs, in the innervated muscle, now consisting of the four different subunits [$\alpha_2\beta\epsilon\delta$], become highly concentrated in the subsynaptic folds underlying the nerve terminal. The gene expression of the four different subunits also declines and becomes restricted to muscle nuclei in the vicinity of the neuromuscular junction (Hall and Sanes, 1993; Corriveau et al., 1995). This change in subunit composition alters the pharmacological and metabolic properties of the AChR and the conductance properties of the ion channel (Sine and Claudio, 1991).

In contrast to muscle, neuronal AChRs are more diverse and are composed of several subunits derived from a family of homologous genes expressed in the nervous system. To date, cloning studies have identified eleven members of that family classified as α-type (α2-α9) and β-type (β2-β4) (Sargent, 1993; Elgoyhen et al., 1994). Coexpression of different neuronal α-subunits with either the neuronal β2 or β4 subunits in *Xenopus* oocytes results in ACh-gated channels with different conductance, open times and burst kinetics (Role, 1992; Patrick et al., 1993). The α7

subunit can form functional homomeric AChRs sensitive to the snake venom, α-bungarotoxin (Bertrand et al., 1992). An α7 transcript in muscle cells, both in culture and in embryonic tissue, has been detected by in situ hybridization; the levels of α7 peaked at E11 and then decline with development. Other types of neuronal AChRs including α 4, α5 and ß4 may also be transiently expressed in developing skeletal muscle (Corriveau wt al., 1995) and may represent incomplete muscle AChR species assembled in the cells and transported to the cell surface (Liu and Brehm, 1993). Alternatively, changes in the combination of the ligand-gated ion channel gene expression may be a general feature of the development; this may explain the single-channel classes observed during muscle development as being neuronal AChRs transiently expressed in the cells (Role, 1992; Corriveau et al., 1995). The nAChR may help mediate other aspects of neuronal development, including the shaping of the neuronal connectivity patterns (Matter et al., 1990; Kilisch et al., 1991). For all AChRs studied so far, expression of the α-subunit is necessary to reproduce ligand binding activity (Leutje et al., 1990; Role, 1992; Sargent, 1993; Patrick et al., 1993). The sequence segment containing Cys_{192} / Cys_{193} contributes to the cholinergic site and is located in a similar position along the α-subunit sequence which suggests that the extracellular domain of all α-subunits folds in a similar manner. Apart from depolarizing the cell membrane, activation of nicotinic receptors at the NMJ also leads to a small, but significant, influx of calcium into the cell (Decker and Dani, 1990). The influx of calcium is voltage dependent and may have important roles in synaptic development, maintenance, and plasticity (Vernino et al., 1994). Moreover, the presence of α 7-containing AChRs in developing muscle might facilitate the early events of synaptogenesis and myoblast fusion due to their ability to increase intracellular calcium levels (Bertrand et al., 1993; Séguéla et al., 1993).

1.2.2. Dystrophin-Associated Protein Complex

Dystrophin and the dystrophin-related protein, utrophin are two members of the spectrin-like molecules localized to the postsynaptic apparatus; both are suggested to form a submembraneous cytoskeleton that interact with extracellular laminin indirectly via a transmembrane complex of proteins, the dystrophin-associated glycoproteins (DAGs) or dystrophin-associated proteins (DAPs) complex, and directly with intracellular actin, thus linking the actin-based cytoskeleton to the extracellular matrix (Ervasti and Campbell, 1993; Tinsley et al., 1994; Campanelli et al., 1994). Nucleic acid sequences for four members of the DAG and DAP complexes have been reported; these include, α-dystroglycan (156-DAG), ß-dystroglycan (43-DAG), syntrophin (59-DAP) and adhalin (50-DAG). Extracellular α-dystroglycan, transmembrane adhalin and intracellular utrophin components of the utrophin-DAG complex codistribute with AChR clusters in response to agrin (Campanelli et al., 1994; Matsumura et al., 1994). Previous work (Campanelli et al., 1994; Gee et al., 1994) indicates that the extracellular DAG, α-dystroglycan, serves as a physiologic receptor for agrin, which in turn mediates AChR clustering.

1.2.3. Dystrophin

Dystrophin, an isoform of ß-spectrin, is present throughout the sarcolemma and in the troughs of the junctional folds; it anchors and stabilizes integral membrane proteins, such as DAGs, which may control calcium flux. Dystrophin links the subsarcolemmal cytoskeleton by binding F-actin through its amino terminal domain and the glycoprotein complex through its carboxy terminal domains (Suzuki et al., 1994). Dystrophin is the product of the X-chromosome linked, 2,300-kilobase gene, an extremely large and complex gene, which regulates the expression of three dystrophin isoforms, a ~427 kDa isoform expressed in brain and muscle and two smaller proteins

of 71 kDa and 116 kDa (Hoffman et al., 1989; Ahn and Kunkel, 1995).

1.3. Syntrophin Association With Neuronal Nitric Oxide Synthase

Syntrophins, a family of dystrophin-associated proteins colocalizes with the neuronal isoform of nitric oxide synthase (nNOS), a nitric oxide (NO) generating enzyme, beneath the sarcolemmal membrane (Ahn et al., 1995). Additional studies indicate that the PDZ motif present in nNOS interacts directly with a similar PDZ consensus domain present in α-syntrophin (Ahn and Kunkel. 1995; Adams et al., 1995) and thus the to the dystrophin complex at the neuromuscular postsynaptic membrane (Brenman et al., 1996) of fast twitch muscle fibers. The PDZ consensus domain, present in structural proteins and enzymes found at specialized cell-cell junctions, is important in protein-protein interactions at the plasma membrane (Cho et al., 1992) and plays a primary role in mediating subcellular localization of nNOS and in turn, assuring its proper expression and interaction with the proper target.

1.4. Skeletal Muscle Pathology

1.4.1. Becker's Muscular Dystrophy and Duchenne's Muscular Dystrophy

Becker's muscular dystrophy (BMD) and Duchenne's muscular dystrophy (DMD) are X-linked recessive diseases characterized by mutations in the DMD gene, resulting in a lack of dystrophin (usually DMD) or the expression of mutant forms of dystrophin (usually BMD) (Hoffman and Kunkel, 1989; Matsumura and Campbell, 1994). Immunohistochemical characterization indicates that DMD selectively affects a subset of skeletal muscle fibers specialized for fast contraction (type II b) (Hoffman et al., 1989; Campbell, 1995). Type IIb fibers respond to the

highest frequency of neuronal stimulation with maximal rates of contraction; they are also the last subset of muscle fibers to develop (Webster et al., 1988). Immunohistochemical studies have further identified an absence of the neuronal form of nitric oxide synthase (nNOS), from skeletal muscle sarcolemma of dystrophic patients, suggesting that the subcellular localization of nNOS is mediated by dystrophin binding (Brenman et al., 1995, 1996; Chao et al., 1996). Furthermore, the translocation of nNOS to the myocyte cytosol may have implications in the pathogenesis of muscular dystrophy (Huang et al., 1994; Brenman et al., 1995, 1996; Chao et al., 1996).

1.4.2. Congenital Myasthenic Syndromes

Congenital myasthenic syndromes are characterized by muscular weakness and fatigue resulting from an impairment of synaptic transmission at the NMJ (Engel, 1984; 1987; Bianca et al., 1994). Electrophysiologic studies (Grob, 1987) reveal that the myasthenic syndrome is a result of one or more of several pre- and post-synaptic abnormalities . These include a reduction in the size of the miniature end plate potentials, a presynaptic abnormality in the resynthesis of ACh or its vesicular storage resulting in lower levels of ACh released at the NMJ (Grob, 1987; Engel, 1987). Postsynaptic abnormalities may include a decrease in the number of AChRs or abnormalities in the receptor kinetics (Conti-Tronconi et al., 1994; Grob, 1987; Beroukhim and Unwin, 1995). Recently, it was reported that changes in AChR kinetics are due to point mutations in the α and ϵ subunit genes of the AChR (Conti-Tronconi et al., 1994; Sine et al., 1995; Ohno et al., 1995). Modification of the receptor channel may lead to a longer lasting depolarization of AChR due to prolonged receptor channel open time. Persistent depolarization is followed by muscle fatigue, weakness and to a progressive and extensive myopathy pronounced myopathy over the long term (Engel, 1984,1987).

1.4.3.Cachexia

Additional skeletal muscle pathologies include a form of muscle wasting, or cachexia, which may occur independent of food intake or malabsorption of nutrients (Buck and Chojkier, 1996). Cachexia is a critical feature of patients afflicted with chronic diseases, including AIDS, cancer and inflammatory disorders. There is evidence for the involvement of tumor necrosis factor (TNF-α)-induced oxidative stress and NOS expression in skeletal muscles of cachectic animals. Moreover, treatment of animals with a NO donor or a superoxide generating system decreases the expression of both myosin, a major structural protein of skeletal muscle, and creatinine phosphokinase (MCK), the enzyme critical for differentiated skeletal muscle function. MCK catalyzes the formation of ATP from phosphocreatine, and is thus important for muscle contraction since the energy required for this process is derived from ATP hydrolysis (Vale, 1994; Buck and Chojkier, 1996). This suggests that NO may mediate the muscle wasting and dedifferentiation induced by oxidative stress in patients with chronic inflammatory diseases, AIDS and cancer. Further evidence to implicate NO in inflammatory and degenerative muscle diseases stems from recent studies to investigate the role of ischemia/reperfusion in muscle injury. These studies indicate that reperfusion-induced edema and increased myeloperoxidase levels (indicative of neutrophil accumulation) were both attenuated in muscles treated with L-NIO, a NOS inhibitor. It is thus suggested that endogenous NO production during ischemia/reperfusion injury may be deleterious to muscle survival (Phan et al., 1996)

1.5. Role of nAChR in Myopathies

Inactivation of cholinesterases at the mammalian NMJ, resulting in excess activation of nAChR, produces extensive muscle necrosis (Fenichel et al., 1972, 1974; Laskowski et al., 1977;

Wecker et al., 1978). The myopathy was totally prevented by prior nerve sectioning (Fenichel et al., 1974; Hudson et al., 1978), by prior administration of hemicholinium depleting the nerve terminal from acetylcholine (Fenichel et al., 1972), and by d-tubocurarine, a nicotinic receptor blocker (Ariens et al., 1969). Myopathic fibers are characterized by an increase in large-diameter vesicles in the cytoplasm under the junctional folds, dilation of the mitochondria, breakdown of sarcoplasmic reticulum and dissociation of Z-disks (Engel et al., 1973; Laskowski et al., 1977; Hudson et al., 1978). This acute form of agonist induced myopathy may involve prolonged calcium influx via the activated nAChR in addition to an increased activity of calcium-activated proteases. This is supported by the previous finding (Leonard and Salpeter, 1982) that inhibition of calcium-activated proteases protects against the agonist induced myopathy in both normal and dystrophic muscle. The mechanisms by which nAChR activation causes degeneration of skeletal muscle cells remain incompletely understood and the putative second messenger(s) has not been identified.

1.6. Involvement of NO in Pathologies

NO has been implicated in a number of skeletal muscle pathologies such as ischemia/reperfusion injury (Phan et al., 1996), Duchenne's muscular dystrophy (Brenman et al., 1995, 1996), Becker's muscular dystrophy (Chao et al., 1996) and spontaneous inflammatory muscle disease (myositis) associated with aging in (Tamir et al., 1996). In the brain, there is enough evidence to suggest that NO may mediate the actions of glutamate acting at the NMDA receptors (Dawson, 1995). NOS is activated by the influx of calcium via NMDA receptors. Overactivation of these receptors however leads to overproduction of NO which in turn results in neurodegeneration associated with ischemic brain damage, stroke and hypoxia (Dawson et al., 1991, 1994; Dawson,

1995; Zhang and Snyder, 1995; Dawson and Dawson, 1996). In this context, it is of interest to determine to what extent NO may be involved in prolonged agonist-induced postsynaptic destruction of skeletal muscle cells, such as, is produced by nicotine or other nicotinic receptor agonists at the NMJ (Leonard and Salpeter, 1979, 1982). Recent data in the literature define and provide an important framework by which we can explore the vast role(s) of NO in different systems. Following is a summary of the biological, physiological, biochemical and pathophysiological functions of NO.

1.7. NO Chemistry

NO is a diatomic, free radical gas. Being of low molecular weight and reasonable hydrophobicity, NO is a highly diffusible compound theoretically able to reach anywhere within cells and tissues (McDonald and Murad, 1996). The discovery of NO as a messenger in the central nervous system has changed the conventional concept of a neurotransmitter. NO is an unorthodox neurotransmitter (Dawson and Dawson, 1996); NO is not stored in synaptic vesicles and is not released by exocytosis upon membrane depolarization. Additionally, NO does not mediate its actions by binding to membrane-associated receptor proteins and its biological actions are neither terminated by enzymatic reuptake nor by enzymatic degradation. However, NO is synthesized on demand and diffuses from one neuron to another where it targets various intracellular components. The activity of NO is terminated after chemically interacting with its target. Unlike the various points of control exerted by the central nervous system in the case of classical neurotransmitters, the key regulation of NO activity is the control of its synthesis by the enzyme NOS.

NO was initially characterized as the "endothelial derived relaxation factor" (EDRF) in 1987

at the Wellcome Foundation; this was a seminal discovery in the area of cardiovascular biology. It was soon discovered that NO is produced by many cell types such as kupffer cells, nonvascular endothelial cells, skeletal muscle cells, kidney macula densa, brain cells and interstitial cells of the penis. NO was subsequently discovered to perform many broad functions including important roles in the immune system and in central and peripheral neurotransmission.

1.8. Enzymology

1.8.1. NOS Isoforms

NOSs generate NO by both constitutive and cytokine/endotoxin induced pathways. Cerebral constitutive NOS, also named neuronal NOS (nNOS) was the first to be isolated and cloned (Bredt and Snyder, 1990; Bredt et al, 1991), revealing a 1433 amino acid 160 KDa protein. The cell membrane located endothelial NOS (eNOS) is 130 KDa (both human and bovine) and exhibits only 58% sequence homology with rat cerebellar nNOS. The inducible NOS (iNOS) is a cytoplasmic protein of about 130 KDa and exhibits 51% sequence homology with rat cerebellar nNOS.

1.8.2. NOSs-Encoding Gene Family

There are at least three genes encoding the NOS family of proteins. The genes for human eNOS, nNOS and iNOS are located on chromosomes 7, 12, and 17, respectively (Marsden et al., 1993, 1994; Chartrain et al., 1994). NOS proteins share 50% amino acid homology indicative of a NOS gene family. Both NOS isoforms, which are dependent upon exogenous calcium and calmodulin, share the highest degree of identity (60%); however within each isoform group, i.e nNOS, iNOS and eNOS, there is a high degree of amino acid identity (80-94%) across species, suggesting that the differences between each group are probably species-related. In spite of the

controversy as to whether the cloned iNOSs are products of a single or multiple genes, available data from amino acid sequence homology studies across various species support the existence of a single iNOS gene (Wood et al, 1993; Xie et al; 1992; Sessa et al; 1994).

1.8.3. Regulation of NO Synthesis

NO is enzymatically synthesized by the stoichiometric conversion of L-arginine to L-citrulline in the presence of oxygen and NADPH through an oxidative-reductive pathway, which involves one of the guanidino atoms of L-arginine and which requires five electrons. NOS utilizes NADPH as an electron donor to catalyze the hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine, an intermediate in the biosynthesic pathway. N^{w}-hydroxy-L-arginine is further oxidized to yield NO and L-citrulline. Although several forms of NOS have been identified, it is clear that all forms purified to date are dependent upon NADPH and molecular oxygen as cosubstrates. Additionally, heme (as protoporphyin IX), flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN) and tetrahydrobiopterin (BH_4) are cofactors for NOS that yield an enrichment in the activity of NOS to varying degrees probably due to the relative binding of these cofactors during purification (Steuhr et al., 1992; Sessa, 1994). Additional cofactors for nNOS and eNOS are calcium and calmodulin; on the other hand, calmodulin was found to be a tightly bound subunit of the macrophage iNOS even though this enzyme is not regulated by external calcium (Bredt and Snyder, 1990; Cho et al., 1992).

1.8.4. Homology of NOS to Cytochrome-P450 Reductase

Cloning and other studies have characterized nNOS and iNOS as cytochrome P-450 type hemeproteins (White et al., 1992; Stuehr et al., 1992), each containing a cysteine thiolate heme prosthetic group which makes the NOS family unique proteins. NOS isoforms are the only known

mammalian proteins that catalyze a hydroxylation reaction (P-450 based reaction) and NADPH reduction (P-450 reductase function) within the same protein, an attribute shared only by the bacterial flavoprotein, Bacillus *megaterium cytochrome* $P\text{-}450_{BM\text{-}3}$ (Ravichandran et al., 1993). In addition, nNOS and iNOS are the only known soluble cytochrome P-450 enzymes.

1.9. NO Biochemistry: Cellular Targets and Effects

1.9.1. Redox Forms of NO

Nearly all cell compartments are possible targets for NO, including proteins, carbohydrates, lipids and nucleic acids (Henry et al., 1993; Stamler, 1994). NO can also interact with biochemical pathways depending on the NO species encountered (Jaffrey et al., 1994; McDonald and Murad, 1996). To address this issue, various valence states of NO have been examined by many investigators. In addition to the free radical form there are the oxidized nitrosonium (NO^+) and the reduced nitroxyl (NO^-) forms of NO which can be generated from NO by physiologically relevant redox mechanisms; these have preferred targets with which they interact (Stamler, 1994; Mohr et al., 1995). However, practical limits exist on the diffusion potential of NO due to its many possible chemical interactions. The greatest factor limiting the availability of NO may be its interaction with the superoxide anion (O_2^-). The other two major interactions limiting NO availability to its intracellular sites of action include binding to transition metals in proteins, such as iron centres in heme proteins, and to proteins containing non heme iron-sulfur centers (Reif et al., 1990; Stamler, 1994). Accordingly, metal- and thiol- containing proteins serve as the major target sites for NO which also constitute the NO- signalling circuitry, and include enzymes, receptors, transcription factors, ion channels and signalling proteins (Brune and Lapentina., 1991; Sessa, 1994; Clementi et

al., 1995) . The thiols or transition metals in these proteins are strategically located at either allosteric or active sites (Mohr at al.,1994)

1.9.2 Reactions of NO species with Heme Proteins

One of the most characterized interactions of NO with transition metals is the iron in the heme of guanylyl cyclase (GC). The NO-iron interaction in GC results in a conformational change in the heme moiety; this activates the enzyme and results in increased cGMP levels which produces smooth muscle relaxation, vasodilatation and inhibition of platelet aggregation principally by decreasing intracellular calcium. NO also interacts with the heme containing enzyme, cyclooxygenase, activating it and leading to an increased prostaglandin synthesis. However, the interaction of NO with the heme moiety leads to the inhibition of the cytosolic enzyme, indoleamine 2, 3- dioxygenase, cytochrome P450 enzymes and NOS itself (Khatsenko et al., 1993; Thomas et al., 1994). Other heme-containing proteins that may be targets for NO include hemoglobin, catalase, myoglobin, cytochrome c and peroxidase.

1.9.3. Reactions of NO Species with Nonheme Iron: Iron Release

In addition to binding to heme proteins, NO can also react with the nonheme iron of iron-sulfur clusters in a number of enzymes. Key iron-sulfur centers inhibited by NO include the mitochondrial electron transport chain, NADH-ubiquinone oxireductase (complexI) and NADH:succinate oxireductase (complexII), the tricarboxylic acid cycle enzyme cis-aconitase and ribonucleotide reductase which synthesizes the deoxynucleosides required for cell replication (Nathan, 1992; Bolanos et al., 1995; Dawson and Dawson, 1996). In contrast to the reactions of NO with heme, the reaction of NO with the iron-sulfur cluster results in the desolution of the cluster (Henry et al., 1993). NO can increase intracellular free iron concentrations by binding to the iron

in both ferritin, the iron storage protein, and to iron-sulfur containing enzymes such as NADH-succinate oxireductase, NADH-ubiquinone oxireductase and cis-aconitase, thus liberating iron that can later cause lipid peroxidation (Reif and Simmons, 1990; Zhang and Snyder, 1995); however, this may be mediated by NO via an iron-sulfur containing protein, iron responsive element binding protein (IRE-BP) also called iron regulatory factor (IRF) which is involved in cellular iron homeostasis. NO disrupts cytosolic aconitase activity, which is converted to iron regulatory factor (IRF) when it loses an iron and is transformed to a protein that binds iron responsive elements (IRE). NO stimulates the IRE-binding function of IRF while diminishing its cytosolic aconitase activity by helping to exposes the mRNA binding site for the transferrin receptor, the erythroid form of 5-aminolevulinate synthase, and ferritin; this permits the binding of (IRF) to the iron responsive element (IRE), decreases the biosynthesis of ferritin and increases intracellular free iron levels (Weiss et al., 1993; Jaffrey et al., 1994; Farell and Blake, 1996).

1.9.4. Reaction of NO with Superoxide Anion: Peroxynitrite Formation

The interaction of NO with $O_2^{\cdot}$ is quite different from that with iron and leads to the production of peroxynitrite ($ONOO^-$) at a rate three times faster than the reaction of superoxide dismutase (SOD) which catalyzes the dismutation of $O_2^{\cdot}$ to hydrogen peroxide. The $ONOO^-$ moiety is a powerful oxidizing agent that may, under certain circumstances, yield nitrogen dioxide (NO_2) and the hydroxyl radical ($OH^{\cdot}$); $OH^{\cdot}$ is considered as the ultimate cytotoxic radical (Beckman et al., 1994a; Radi et al., 1991, 1994). Both $ONOO^-$ and $OH^{\cdot}$ contribute to the cytotoxicity of activated macrophages and neutrophils and may also mediate tissue injury, acute inflammation, hypoxic-reperfusion injury, hypoxia, glutamate mediated neurotoxicity, atherosclerosis, acute endotoxemia, and other inflammatory bowel syndrome (Beckman et al., 1994b; Lin et al., 1995) . The free radical

moeity ($ONOO^-$) reacts readily with sulfhydryls and with zinc-thiolate moieties, in addition, it can nitrate and hydroxylate aromatic rings on amino acid residues, oxidize lipids, proteins, and DNA (Radi et al., 1994b; Simonian and Coyle, 1996).

1.9.5. Reactions of NO with Thiols

S-Nitrosylation of many proteins is emerging as an important system utilized by NO to regulate many important biological functions (Stamler, 1994). NO stimulates auto-ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dawson, 1995; Dawson and Dawson, 1995; Mohr et al., 1996). NO depresses glycolysis by interacting with the active site cysteine in GAPDH thus inhibiting its catalytic activity. Through the formation of S-nitrosoglutathione, NO can activate the hexose-monophosphate shunt pathway by depleting intracellular glutathione levels. Thioester long chain fatty acid acylation of neuronal proteins is also inhibited by NO through the modification of cysteine thiols in the neuronal growth cone (Hess et al., 1993). NO can also reduce excitatory neurotransmission by binding to the redox site and decreasing receptor activity (Lipton et al., 1993; Simonian and Coyle, 1996).

1.10. Physiological Roles of NO

Since the first demonstration of nitrergic transmission and the fact that endothelium-derived factor (EDRF) was NO, hundreds of papers have shown the wide distribution of NO in the autonomic and enteric systems. The finding that nNOS possesses diaphorase activity (Hope et al., 1991; Dawson et al., 1991) provide a simpler method for histochemical detection of NOS using the reduction of nitro blue tetrazolium (NBT) to formazan as the marker. Immunohistochemical staining has also helped localize central and peripheral immunoreactive nNOS neurons. However,

physiological function of nitrergic neurons was provided by reflex activation of specific neural pathways and by the application of nitromimetics such as organic nitrites (e.g. amyl nitrite), which are NO-donating nitroso-alcohols and nitrosothiols (e.g. S-nitrosocysteine) (Rand and Li, 1995).

Under physiologic condition, NO plays a role in a variety of functions, including synaptogenesis, a mediator of synaptic plasticity in the hippocampus and cerebellum, memory formation, regulation of cerebral blood flow, and neuroendocrine secretion (Hess et al., 1993; Lin and Bennet, 1994; Pogun et al., 1994; Dawson and Dawson, 1995; Peunova and Enikolopov, 1995; Gross and Wolin, 1995; Farell and Blake, 1996). NO was first recognized as a physiologic messenger molecule that mediates the regulation of blood vessel tone and attenuates vasoconstriction *in vivo* and *ex vivo*. NO also regulates tumoricidal and bactericidal actions of macrophages and functions as a non-adrenergic, noncholinergic neurotransmitter (NANC) involved in gastrointestinal smooth muscle relaxation and peristalsis, pulmonary vasodilatation, and penile erection (Sanders and Ward, 1992; Thomas et al., 1994; Konturek and Konturek, 1995; Rand and Li, 1995; McCann and Rettori, 1996; McDonald and Murad, 1996). NO is also emerging as an important endogenously derived neurotoxin that may contribute to neuronal cell death in a variety of disorders of the nervous system (Montague et al., 1994; Dawson and Dawson, 1995; Shultz et al., 1995; Zhang and Snyder, 1995).

1.10.1. Vasculature

In the vasculature, NO produced by eNOS in the adventitial layer of blood vessels is a principal determinant of resting vascular tone. NO is produced in the endothelium at a basal rate, which increases in response to agonists that increase intracellular calcium and, in turn, activate nitric oxide synthase. The physiological stimuli of the release of nitric oxide include the biochemical

mediators thrombin, adenosine diphosphate, and bradykinin; the physicochemical mediators include shear stress and cyclic strain. NO diffuses from endothelial cells to smooth muscle cells where it induces smooth muscle relaxation by activating GC. Furthermore, NO inhibits the adhesion, activation and aggregation of platelets (Thomas et al., 1994) and thereby confers an important antithrombotic property on the endothelial surface. In early atherosclerosis, NO dependent vasorelaxation is impaired at the atheroma site. In established disease, however, NO release is impaired throughout the atherosclerotic coronary vessel and not just at sites of atheroma (Beckman 1994b). Additionally, evidence suggests a role for NO as an antimitogenic agent in vivo, thus preventing excess vascular smooth muscle proliferation which is a hallmark of atherosclerosis (Dominiczak and Bohr, 1995).

Studies indicate a role for NO in mediating increased blood flow to skeletal muscle during exercise. Experiments on exercised muscle indicate that NO may be involved in the regulation of vascular tone in both skeletal muscle and cardiac muscle by enhancing metabolic vasodilation and via regulation of mitochondrial oxygen consumption (Shen et al., 1995). Studies have also demonstrated that NO-dependent mechanisms mediate the actions of insulin-like growth factor-I (IGF-I), which exerts both a growth-like increase in protein synthesis and an insulin-like decrease in protein degradation. NO mediates the increase in blood flow induced by IGF-I and is also involved in the response of muscle to growth hormone-like IGF-I (Fryburg, 1996).

1.10.2. Autoregulation of Blood Pressure: Central Effects of NO

Multiple studies link NO to the long term regulation of peripheral vascular resistance. Many laboratories have been investigating the potential mechanisms of action of NO in the central nervous system action or alterations in renal homeostasis (Nakamoto et al., 1995). Considerable data suggest

that NO causes a decrease in sympathetic nerve activity and blood pressure and participates in the maintenance of resting cerebrovascular tone. Recent work has shown that NO donors administered intracerebroventricularly cause a fall in mean arterial blood pressure while intracerebroventricular injections of NOS inhibitors cause a pressor response. These observations imply that NO acts as an inhibitory neurotransmitter in cerebral arteries. Furthermore, evidence suggests that failure of cerebrovascular autoregulation during marked increase in cerebral perfusion pressure is caused by the release of NO from nerves of the sphenopalatine ganglion (Talman and Dragon, 1995), which causes relaxation of arterial smooth muscle cells (Dominiczak and Bohr, 1995).

1.10.3. Renovascular Hypertension

Recent studies on renovascular hypertension indicate that chronic inhibition of NOS results in the evolution of hypertension due to chronic renal ischemia produced by a decrease in glomelular filtration rate, and an increase in glomelular capillary pressure. Nakamoto et al. (1995) have recently demonstrated an important contribution of NO in modulating the increased activity of the peripheral renin-angiotensin system during the evolution of renovascular hypertension, suggesting that both NO and angiotensin-(1-7) mechanisms act in synergy to buffer the increase in vascular resistance produced by chronic renal ischemia.

1.11. NO in the Central Nervous System

1.11.1. Synaptic Plasticity

NOS is present in many areas in the brain including the cerebellum, olfactory bulbs, caudate-putamen and the hippocampus (Bredt et al., 1990b; Farrell and Blake, 1996). One of the key research interests in the CNS has been to understand the role of nNOS in neurotrnsmitter release in

the hippocampus and, in particular, its role in long term potentiation (LTP) (Bennett, 1994). Cerebral arteries are innervated by postganglionic NO containing fibers that arise from the sphenopalatine ganglion and pass through the ethmoid foramen. NO plays a role in either the initiation or the maintenance of LTP at synapses in the autonomic ganglia (Bennett, 1994; Garthwaite and Boulton, 1995). LTP is initiated when a neuron receives several simultaneous signals which trigger the NMDA class of glutamate receptors and lead to an increase in intracellular calcium levels. This leads to "potentiation", which is central to some forms of learning and memory. Exogenous application of nitric oxide donors to hippocampal slices increases the efficacy of transmission. This is due to NO increasing the size of synaptic potentials during LTP as a consequence of an increase in the secretion of quanta (Lin and Bennett, 1994). Several lines of evidence suggest that the presynaptic neuron may also participate in the longer lasting aspects of LTP, known as maintenance and expression. Thus, LTP is induced postsynaptically but may be expressed, at least in part, presynaptically. This shift of locus requires that the presynaptic cell receive a signal from the postsynaptic cell for LTP to occur. The postsynaptically generated retrograde signal would then be responsible for the increase in neurotransmitter release (Benenett, 1994; Lin and Bennett, 1994). Recently, several labs (Schuman and Madison, 1994; Garthwaite and Boulton, 1995) have established that NO is the retrograde messenger in LTP; this is further supported by the calcium/ calmodulin dependence of the nNOS and the established role for both molecules in LTP. Furthermore, blockade of LTP by extracellular application of the NO quencher, hemoglobin, and by NOS inhibitors injected into the postsynaptic neuron is consistent with the idea that NO functions as an intracellular signal, travelling from the post- to the presynaptic neuron (Bennett, 1994; Lin and Bennett, 1994; Schuman and Madison, 1994; Garthwaite and Boulton,

antagonist, inhibits the increase in NOS activity, while both pregnancy and estradiol increase the amount of mRNAs for both NOS isoforms and for nNOS in skeletal muscle (Weiner et al., 1994; McCann and Rettori, 1996).

1.12.2. Gastrointestinal (GI) Tract

The role of NO in NANC neurotransmission has been investigated most extensively in the gut. nNOS immunoreactivity has been detected in a subpopulation of myenteric and submucosal neurons in proximal colon. eNOS immunoreactivity, however, can be identified in the endothelium of small intramural blood vessels and in the interstitial cells of the gastrointestinal (GI) smooth muscle that act as pacemaker cells for GI muscles by initiating phasic depolarizations of the smooth muscle cell syncytium (Shuttleworth and Sanders, 1996). NOS is localized in fibers and varicosities of the inhibitory motor neurons that course through the muscle layers. Interstitial cells and enteric inhibitory neurons not only possess the ability to synthesize NO, they may also recycle the byproduct, citrulline, back to arginine, thus sustaining inhibitory neurotransmission (Shuttleworth and Sanders, 1996). The reduction in mechanical activity resulting from the stimulation of enteric inhibitory neurons is produced by an enhancement of calcium entry into varicosities which will in turn activate NOS. NO signals are transduced by GC, the production of cGMP, activation of protein kinase G, and direct stimulation of cellular effectors such as K+ channels. NO-dependent neurotransmission in the GI tract is critical for the relaxation of sphincters, gastric accommodation, receptive relaxation during feeding, and the descending inhibition arc of peristaltic reflex (Sanders and Ward, 1992; Shuttleworth et al., 1995; Konturek and Konturek, 1995).

1.12.3. Bronchodilatation

NO mediated NANC nerve bronchodilatation is also the sole neural mechanism of airway dilatation in man. Inhaled NO is a pulmonary vasodilator that reverses acute hypoxic pulmonary vasoconstriction in vivo (Sanna et al., 1994; Giaid and Saleh, 1995). Comparative studies indicate that eNOS expression is highly abundant in normal lungs; the enzyme expression, however, is diminished in the endothelium of pulmonary arteries of patients with pulmonary hypertension. This diminution correlates inversely with the total pulmonary resistance in patients with plexogenic pulmonary arteriopathy (Giaid and Saleh, 1995).

1.12.4. Penile Erection

Several lines of study indicate a role for NO in penile erection (Burnett et al., 1993; Lugg et al., 1995). Penile erection results when cavernosal and pelvic nerves induce vasodilation of arteries that supply the corpora cavernosa in response to NANC neurotransmission (Rajfer et al., 1992; Burnett et al., 1993). There is considerable evidence supporting the role of NO in penile erection as both a neurotransmitter synthesized and released from the pelvic plexus into the corpus cavernosum, and as a vasodilator which relaxes the corpus cavernosum allowing the corpora to fill with blood and swell, producing an erection. Additional studies using electrical field stimulation of rat cavernosal nerve indicate that aging in the rat induces erectile dysfunction associated with a decrease in the levels of penile NOS (Lugg et al., 1995). Studies have also indicated that sex hormones induce the production of the calcium-dependent nNOS (Weiner et al., 1994; Lugg et al., 1995).

1.13. NO in Skeletal Muscle

1.13.1. Distribution of NOS in Skeletal Muscle

Both constitutive isoforms of NOS, eNOS and nNOS, are expressed in normal skeletal muscle (Kobzik et al., 1994, 1995). Immunohistochemical staining have localized nNOS to the sarcolemma of fast-twitch muscle fibers or type II fibers (Kobzik et al, 1994) where it influences skeletal muscle contractile activity. Approximately 80% of nNOS in mouse skeletal muscle partitions with the particulate membrane fraction (Hecker et al., 1994). Differential fractioning indicates that nNOS anchors to the skeletal muscle cytoskeleton by a 230 amino acid N-terminal unique to nNOS among the NOS family (Brenman et al., 1995). The unique N-terminus of nNOS present in skeletal muscle contains a 66 amino acid motif (a GLGF/PDZ motif) homologous to a heterogenous family of signalling enzymes localized to specialized cell-cell junctions.

1.13.2. Post-translational Modification of nNOS in Skeletal Muscle

Recently, Silvagno et al. (1996) have identified a novel nNOS mu isoform which is 102-base pair (34 a.a) larger than that expressed in the cerebellum and functions exclusively in the differentiated muscle. The expression of nNOS mu occurs at the stage of myotube fusion in culture indicating a function for NO as a molecular modulator in numerous processes of cellular development and physiology. Studies indicate that post-translational modification of nNOS in skeletal muscle allows for a tissue-specific regulation of its activity (Bredt, 1996; Silvagno et al., 1996).

1.13.3. nNOS Associates with the Dystrophin Complex in Skeletal Muscle

In skeletal muscle, nNOS is associated with the sarcolemma (Kobzik et al., 1994) by a direct binding to α1-syntrophin (Chao et al., 1996; Brenman et al., 1996); a protein associated with the

cytoplasmic side of the AChR and a member of DAPs (Gee et al., 1994; Matsumura and Campbell, 1994; Tinsley et al., 1994). α1-syntrophin is involved in the formation/stabilization of AChR clusters linking agrin in the extracellular matrix and utrophin within the cell. to establish the junctional region between the motor neuron and muscle (Tinsley et al., 1994).

1.13.4. Physiologic Modulation of Skeletal Muscle Activity

Recent observations have identified NO as a physiological modulator of skeletal muscle. Contrary to the role of NO, reactive oxygen intermediates (ROI) present in unfatigued muscle have a positive effect on excitation-contraction coupling and are optimal for maximal contractile activity (Reid et al., 1993). In the resting muscle, NO inhibits force by increased production of cGMP which is associated with low rates of ROI generation. On the other hand, muscular exertion in the actively contracting state results in an increased production of reactive oxygen intermediates which diverts NO from the heme centre in guanylate cyclase towards the sulphydryl centers on the sarcoplasmic reticulum (Reid et al., 1993; Stamler, 1994). Sufficient perturbation of the cellular redox state resulting from NO-mediated S-nitrosylation and thiol oxidation reactions would then promote intracellular calcium release and an augmentation of muscle contractile performance and force production (Reid et al., 1993; Kobzik et al., 1994).

1.13.5. Role of NO in Skeletal Muscle Cell Energy Metabolism

Studies suggest a role for NO in regulating basal glucose metabolism in rat skeletal muscle preparations (Balon and Nadler, 1994). NOS inhibitors diminish basal 2-deoxyglucose transport by 25%, while an increased NO production is associated with an increased glucose uptake (Albina and Mastrofrancesco, 1993). eNOS is densely expressed within mitochondria-rich fibers throughout the myocyte cytoplasm where it can modulate mitochondrial respiration (Kobzik et al., 1994). NO

inhibits mitochondrial oxygen consumption, thus mitochondrial respiration, via interactions with iron-containing enzymes such as cytochrome c and cytochrome a (Cleeter et al., 1994; Brown, 1995; Bolanos et al., 1995).

1.13.6. Targets of NO in Skeletal Muscle:

NO could affect skeletal muscle activity via two mainly identified mechanisms. One is related to activation of GC which decreases the force generation by skeletal muscle fibers via an increased production of cGMP (Kobzik et al., 1994). The other involves a possible S-nitrosylation of thiol groups in the sarcoplasmic reticulum such as the calcium pump (Meszaros et al., 1996; Wolosker et al., 1996). The rate of calcium release assessed from the ryanodine receptor in the sarcoplasmic reticulum of skeletal muscle homogenate is reduced by NO generators. Thus, the effect of NO on the ryanodine receptor, the principal calcium release pathway in skeletal muscle, may explain the NO-induced depression of contractile force (Meszaros et al., 1996).

1.14. Pathophysiological Effects of NO

Deranged NO synthesis is the basis for a number of pathophysiological states such as atherosclerosis, pulmonary hypertension, pyloric stenosis, renal failure-associated hypertension. Excess nNOS production has been implicated in animal models of stroke (Huang et al., 1994; Estevez et al., 1995; Gross and Wolin, 1995) while iNOS plays a role in the destruction of pancreatic ß-cells (Kaneto et al., 1995), rheumatoid arithritis and inflammatory disorders including septic shock (Farell and Blake, 1996; Lowenstein et al., 1994).

1.14.1. Involvement of NO in Neurodegeneration: Conflicting Results

NO and related species appear to play a dual role, having both detrimental and protective

effects. However, even when the participation of NO in neurodegeneration is indicated, the precise role is not fully understood (Strijbos et al., 1996). NO or $ONOO^-$ could participate in cell death via various mechanisms (Bolanos et al., 1995; Gross and Wolin, 1995; Troy et al., 1996). It has been suggested that the real culprit in NO mediated toxicity may be $ONOO^-$. Under conditions in which SOD is decreased, or NO and O_2^- are increased, the formation of $ONOO^-$ is favoured (Greenlund et al., 1995). Recent studies suggest at least three reasons for these controversial and confusing results. First, the redox state of the cell may determine the predominant NO species and thus the cellular effects of NO. Under reducing conditions, NO free radical is formed and can interact with O_2^- to produce $ONOO^-$. Under oxidizing conditions, the NO ion, NO^- is formed and can reduce excitatory neurotransmission through the NMDA receptor by binding to the redox site and decreasing receptor activity (Lipton et al., 1993; Hewett et al., 1994). Second, the percentage of NOS-containing neurons in primary cultures may influence toxicity in vitro; NOS-containing neurons seem to be insensitive to NO toxicity in cerebral granule cells and a subset of cortical neurons. Third, differential inhibition of neuronal versus endothelial NOS activities may also contribute to the dual protective and degenerative role of non-selective NOS inhibitors. Inhibition of both nNOS and eNOS decreases cerebral blood flow and thus masks any possible cytoprotective role for nNOS. Knockouts of nNOS mice are protected against ischemic brain damage, whereas the additional loss of eNOS potentiates injury; these data suggest that nNOS production exacerbates acute ischemic injury, whereas eNOS protects after middle cerebral artery occlusion (Huang et al., 1994; Panahian et al., 1996).

1.14.2. Role of NO in Neurodegeneration

Exposure to NO induces several forms of neuronal injury including hypoxic-ischemic

damage (Estevez et al., 1995; Troy et al., 1996). It has been demonstrated that mice with a nNOS gene knockout are resistant to NMDA glutamate receptor-induced focal and transient global ischemia (Huang et al., 1994; Schulz et al., 1995; Panahian et al., 1996). The best established candidate for mediation of neurotoxicity by NO is poly (ADP-ribose) synthetase (PARS). PARS is a nuclear enzyme which utilizes NAD as a substrate to attach 50-100 ADP-ribose units of ADP-ribose to nuclear proteins, four ATP molecules are consumed in the process of one NAD molecule regeneration. Following NO-induced DNA damage , PARS is activated in brain tissue (Huang et al., 1994), depleting neuronal cells of NAD and ATP and thus of their available energy stores. Furthermore, in addition to blocking NMDA-induced neurotoxicity in cortical cultures, PARS inhibitors protect CA1 neurons and dorsal horn spinal neurons from injury mediated by NO and oxidative stress (Zhang and Snyder, 1995). Alternatively, NO or peroxynitrite could cause the release of glutamate (Montague et al., 1994) and/or inhibit glutamate uptake (Pogun et al., 1994) and thereby raise the extracellular glutamate concentration and promote damage through other mechanisms which may possibly be NO-independent mechanisms (Strijbos et al., 1996).

1.14.3. Role of NO in Skeletal Muscle Cell Injury

Application of either S-nitrosoglutathione (GSNO) or S- nitroso N-acetyl penicillamine (SNAP), two NO donors, inhibited the activity of the sarcoplasmic-reticulum bound creatine kinase by a mechanism which involves nitrosylation of critical sulfhyryl (SH) groups of creatine kinase which affects ATP regeneration (Wolosker et al., 1996). Consequently, NO may be involved in mechanisms of skeletal muscle cell injury via the inhibition of creatine kinase in skeletal muscle cells. Recent findings suggest that endogenous NO production during ischemia / reperfusion injury may be deleterious to muscle survival (Phan et al., 1996) which is in line with previous reports

indicating that NO reversibly inhibits mitochondrial respiration by competing with oxygen at cytochrome oxidase (Bolanos et al., 1995; Brown, 1995; Kobzik et al., 1995). Viner and coworkers (1996) found that skeletal muscle in aged rats show increased relaxation times which is correlated with a dysfunction of the sarcoplasmic reticulum (SR) Ca-ATPase in SERCA2 a slow-twitch isoform of the Ca-ATPase. This indicates that a less effective antioxidant defence system of aged organisms results in a higher accumulation of nitrated SERCA2a by peroxynitrite ($ONOO^-$) formed under conditions of simultaneous generation of superoxide ($O_2^{\cdot-}$) and NO (Viner et al., 1996).

1.15. Concluding Remarks

In conclusion, NO and its reactive species may initiate a series of damaging biochemical events such as lipid peroxidation, protein oxidation, and oxidation of thiols, which may result in activation/deactivation of enzymes with a prosthetic iron group, depletion of ATP, NADH and NADPH, the inability to maintain normal ion gradients and damage to calcium and other ion transport systems (Phan et al., 1994; Tamir et al., 1995). However, it remains to be determined if increased activity of NOS in damaged tissues and dystrophic muscle is of primary importance or secondary to a fundamental preexisting defect or malfunction. It would therefore be important to decipher the potential role(s) of NO, in the absence or presence of receptor agonists, in the control of the cell death in various tissues. This control may as well be a programmed process of cell death involved in normal skeletal muscle development or a genetic predisposition to myopathic conditions.

1.16. Statement of the Problem

As discussed in the Introduction, several lines of study have identified NO as a molecule with dual functions. Analysis of the role of NO in different tissues has established its importance as a mediator molecule with both cytoprotective as well as cytotoxic properties.

Recent studies to elucidate the role of NO in skeletal muscle, have demonstrated that nNOS is critical for skeletal muscle structure, function and regulation. While these studies have provided information on the role of NO in the physiology and pathology of skeletal muscle, none has explored a possible role for NO in nicotine induced muscle cell degeneration. Emerging evidence suggests a role for the nicotinic receptor at the neuromuscular junction in cellular development, growth and degenerative processes. Previous work has demonstrated that overexposure of muscle cells to nicotinic agonists or the inhibition of acetylcholinesterase may result in the necrosis of muscle cells. The degenerative effects of nicotine was prevented by calcium chelators, indicating a role for calcium in the myopathy. Nonetheless, no further investigations were carried to elucidate the subsequent intracellular messengers.

On the basis of preliminary work, **the overall objective of my study was to determine whether NO mediates nicotinic receptor-induced myopathy in cultured skeletal muscle cells and to further investigate the underlying mechanisms involved.**

2.0. INVOLVEMENT OF NITRIC OXIDE IN NICOTINIC RECEPTOR MEDIATED MYOPATHY

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2.1. SUMMARY

Previous studies have shown that nicotinic cholinergic agonists induce muscle cell degeneration. Although an involvement of calcium is well documented, subsequent intracellular steps have not been identified. The present experiments test whether nitric oxide (NO) may play such a role. Both the irreversible NOS inhibitor L-^{5}N-iminoethyl ornithine (NIO) and L-nitroarginine methyl ester (L-NAME), a reversible inhibitor, protected the muscle cells from the myopathic effects of nicotine. These results may suggest that nicotinic receptor stimulation produces an increase in NO which results in muscle cell degeneration. In line with this interpretation, exposure of the muscle cultures to the NO donor sodium nitroprusside (SNP) resulted in a dose dependent decline in myotube branch points. Neither NIO nor SNP altered the binding of the nicotinic receptor agonist ^{125} I-α-bungarotoxin (^{125}I-α-BGT) to muscle cells in culture, indicating that the effect of these agents was not mediated through an interaction at the nicotinic receptor. The present results suggest that nicotinic receptor activation results in skeletal muscle degeneration through an increase in NO production.

2.2. INTRODUCTION

NO is a short lived ubiquitous molecule which serves as a mediator of vasodilatation (Moncada et al., 1991), platelet aggregation (Radomski et al., 1990a; Lowenstein et al., 1994) and cellular toxicity (Dawson et al., 1991; Xie et al., 1992; Schulz et al., 1995; Dawson, 1995; Przedborski et al., 1996; Schulz et al., 1996). NO also mediates transmission in the central and peripheral nervous system (Dawson et al., 1992; Snyder, 1992; Garthwaite and Boulton, 1995), where it has been implicated in long term potentiation and depression (Chapman et al., 1992; Lin and Bennett, 1994; Dinerman et al., 1994; O'Dell et al., 1994). NO is synthesized from L-arginine by the enzyme NO synthase (NOS). Three distinct isoforms of NOS have been identified to date. These include two constitutive, Ca^{++}/calmodulin dependent gene products, of which one is neuronal NOS (nNOS), a cytosolic enzyme, highly concentrated in brain, neurons of the myenteric plexus, kidney macula densa (Huang et al., 1993) and skeletal muscle (Kobzik et al., 1994). The second constitutive isoform, endothelial NOS (eNOS), is a membrane bound enzyme (Robinson et al., 1995) present in the endothelium of blood vessels, the epithelium of many tissues including the bronchial tree, the pyramidal cells of the hippocampus (O'Dell et al., 1994) and in skeletal muscles (Kobzik et al., 1995). The third NOS isoform, the inducible NOS (iNOS), is a cytosolic, Ca^{++} independent enzyme. iNOS is transcriptionally regulated and is activated by certain cytokines and endotoxin lipopolysaccharides; this form is present in nearly all nucleated cells including macrophages, kupffer cells, endothelial cells, fibroblasts, vascular smooth muscle cells and mesangial cells (Radomski et al., 1990b; MacMicking et al., 1995)

Acetylcholine released at the neuromuscular junction mediates its effects by interacting with

the muscle-type nicotinic receptor, one of the best characterized ligand gated ion channel receptor. In addition to its classical role in neurotransmission, acetylcholine has been shown to affect a variety of growth related functions ranging from developmental to degenerative processes (Ariens et al., 1969; Fenichel et al., 1972; Leonard and Salpeter, 1979, 1982; Mattson, 1989; Quik, 1995). In analogy to the neurotoxic actions of excitatory amino acids at their receptors (Meldrum et al., 1990; Choi, 1990; Dawson et al., 1991), nicotinic acetylcholine receptor activation may also result in damage to the endplate region of skeletal muscle. Inhibition of acetylcholinesterase, which increases acetylcholine levels at the neuromuscular junction produces an extensive muscle necrosis (Fenichel et al., 1972; Engel et al., 1973; Freeman et al., 1976). The severity of the myopathy was decreased by prior nerve sectioning, depletion of acetylcholine within the nerve terminal by the acetylcholine transport inhibitor hemicholinium or application of the nicotinic receptor blocker d-tubocurarine, suggesting that the response is mediated by acetylcholine through an interaction at the receptor (Ariens et al., 1969; Fenichel et al., 1972; Hudson et al., 1978). *In vitro* studies have shown that application of the cholinergic agonist carbachol resulted in a calcium dependent muscle damage which was prevented by nicotinic receptor blockade (Leonard and Saltpeter, 1979, 1982). These studies provide evidence for a myopathic/degenerative role mediated by excess acetylcholine released at the neuromuscular junction.

Interestingly, excitatory amino acids such as glutamate exert neurotoxic effects at their receptors, which are mediated by an initial flux of calcium into the cell (Meldrum and Garthwaite, 1990; Garthwaite and Boulton, 1995). Accumulating evidence now suggests that glutamate receptor activated neurotoxicity is mediated by NO (Dawson et al., 1991; Reif, 1993; Zhang et al., 1994; Dawson, 1995; Schulz et al.,1995). These observations raised the question whether NO may be

involved in acetylcholine induced muscle cell degeneration. nNOS is present in skeletal muscle (Nakane et al., 1993; Kobzik et al., 1994, 1995). Studies also show that NO modulates muscle contractility and relaxation (Kobzik et al., 1994, 1995) and is involved in activity dependent synaptic suppression during development at the neuromuscular synapses (Wang et al., 1995). As well, NO directly modulates mitochondrial function, the mitochondrial electron transport chain (Schweizer et al., 1994) and oxygen consumption in intact skeletal muscle (King et al., 1994).

The present experiments were done to determine whether NO is involved in agonist induced myopathy at the neuromuscular junction. For this purpose, neonatal muscle cells in culture were exposed to nicotine in the absence or presence of NOS inhibitors, as well as drugs which release NO. The results are the first to suggest that NO is a second messenger which may mediate the degenerative effects of nicotinic receptor activation in skeletal muscle.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Cytosine arabinose (cytosar), d-tubocurarine, bovine serum albumin (BSA), L-nitroarginine methyl ester (L-NAME), D-nitroarginine methyl ester (D-NAME), were purchased from Sigma Chemicals, St. Louis, MO. Sodium nitroprusside (SNP) was obtained from Fisher, Montreal, Quebec, L-^{5}N-iminoethyl ornithine (NIO) from Cayman, Ann Arbor, MI, and nicotine hydrogen (+)-tartrate from BDH Ltd., Poole, England. Trypsin, medium 199, minimal essential medium, horse serum, penicillin and streptomycin were purchased from Gibco/BRL, Grand Island, NY and ^{125}I-α-bungarotoxin (^{125}I-α-

BGT), 10-20 μCi/μg, from New England Nuclear, Boston, MA.

2.3.2. Muscle cell culture

Rat myotube cultures were prepared, under sterile conditions from 1-2-day old Sprague Dawley rats (12-16 pups) as previously described (Braun et al., 1989) with some modifications. Minced muscle from the pectoralis and hind limb was washed in phosphate-buffered saline containing 0.5 mM Mg^{++}. The muscle tissue was dissociated for 45 min in 10 ml 0.25% trypsin, during which time the cells were gently mixed with a magnetic stirrer. Ten ml 0.25% trypsin was then added and the cells allowed to dissociate for a further 30 min. An equal volume of Hank's balanced salt solution (magnesium and calcium free) was added to the cell suspension which was centrifuged for 10 min at 1000 x g. The supernatant was discarded and the pellet resuspended in culture medium which consisted of 65% minimal essential medium, 25% medium 199, 10% horse serum, penicillin (50 U/ml) and streptomycin (50 μg/ml).

For the experiments involving morphological studies, cells were plated onto 35 mm collagen-coated dishes (Nunc) at a density of 1.1-1.3 million cells/dish. After 3 days in culture, the medium was replaced with one containing 10 μM cytosar to limit the proliferation of fibroblasts. Cytosar was removed after one or two days and the cells were maintained in regular culture medium to which various concentrations of the drugs were added.

For the ^{125}I-α-BGT binding studies, cells were plated onto collagen-coated 24-well multiwell plates at a density of 0.30-0.35 million cells/well. Cultures were incubated in a humidified atmosphere of 5% CO_2 /95% air. Cells were not maintained for more than 8-10 days after plating since, with increasing myotube development, enhanced contraction caused cells to lift from the culture plates.

2.3.3. Assessment of myotube branch formation

Numerical analysis of myotube branching was done at various times after plating using phase contrast microscopy. Previous studies (Quik et al., 1992) had shown that determination of the number of myotube fusion or branch points provided a good index of muscle cell viability; declines in the number of branch points were associated with a corresponding reduction in muscle length and nicotinic receptor binding. To count the number of myotube branch points, a diametric strip was counted at 100x magnification for each culture plate. The strip represented approximately 12-15 fields per plate, with the number of myotubes ranging from 3-30 per field depending on the condition to which the cells were subjected. Each culture condition was tested in quadruplicate.

2.3.4. ^{125}I-α-BGT binding to neonatal muscle cells in culture

Radiolabeled α-BGT binding to neonatal muscle cells was done as described (Quik *et al.*, 1992). Prior to the binding assay the cells were washed twice with 2 ml Dulbecco's modified Eagles medium (DMEM) containing 1 mg/ml BSA. Cells in culture were preincubated for 60 min at 37°C in DMEM containing 10 mg/ml BSA in the presence or absence of the indicated drugs. This was followed by a 90 min incubation at 37°C in the presence of ^{125}I-α-BGT (1.8 nM). Binding was terminated by removal of the medium followed by four 1 ml washes with DMEM containing 0.1% BSA. The cells were then resuspended in 0.5 ml 1.0 N NaOH and the radioactivity determined using a gamma counter. Nonspecific binding was defined as the binding in the presence of 10^{-4} M d-tubocurarine, a concentration which results in a maximal inhibition of ^{125}I-α-BGT binding.

2.3.5. Statistics. Statistical analysis were done on INSTAT software using two-way analysis of variance (ANOVA).

2.4. RESULTS

2.4.1. Effects of nicotine on myotube morphology

Previous time-course experiments have established that a 24-48 h exposure period is needed to detect the nicotine-induced effects on myotubes. A longer exposure time however, would result in a complete degeneration of muscle cells in culture. Figure 1 shows an example of nicotine-induced muscle cell degeneration; 3 x 10^{-5} M nicotine (fig. 1B) markedly decreased cell size and the number of branch points in the differentiated myotubes as compared to the control untreated cells (fig. 1A). Muscle cell degeneration was characterized by regression of myotubes with subsequent detachment of the myotube branches from each other and/or the culture dish. The percentage decrease in myotube branch points may vary between different experiments. Various explanations are open and include differences in the state of the cultured cells, the effectiveness of cytosar in eliminating fibroblasts, a variability in the incubation period between the different cultures studied, and the changes accompanied with the use of a different batch of horse serum.

2.4.2. Effect of nicotinic antagonists on the nicotine induced myopathy

To determine whether the nicotine-induced muscle degeneration was due to a specific interaction at nicotinic receptors, the effect of the nicotinic receptor antagonist d-tubocurarine was examined. Muscle cultures were preincubated with 10^{-4} M d-tubocurarine for 2 h before nicotine addition. Twenty-four h later, muscle cell viability (branching) was assessed using phase contrast

microscopy. Figure 2 illustrates that nicotine resulted in a dose-dependent decline in the number of muscle branch points which was statistically significantly different from control at nicotine concentrations of 10^{-6} M, 10^{-5} M, 3 x 10^{-5} M and 10^{-4} M (*P<0.05, **P<0.01). The nicotinic antagonist d-tubocurarine (10^{-4} M) completely prevented the degenerative effects of nicotine on the muscle cells in cultures, indicating that the effect of nicotine was receptor mediated.

2.4.3. Effects of NOS inhibitors on nicotine-induced muscle cytotoxicity

To test whether NO is involved in the nicotine-induced effects on the muscle cultures, the irreversible NOS inhibitor NIO was added to the muscle cultures in the presence or absence of nicotine. The degree of branching was assessed after 24 h. NIO, at either 3 x 10^{-5} M or 10^{-4} M, did not significantly affect myotube branching when compared to the control untreated condition (fig. 3). As previously shown, nicotine at 3 x 10^{-5} and 10^{-4} M induced a decrease in the myotube number of branch points. On the other hand, when cultures were exposed to both nicotine and NIO (3 x 10^{-5} M and 10^{-4} M), the nicotine induced myopathy was partially prevented (fig. 3).

The results of the experiments with NIO prompted us to test the effects of the reversible NOS inhibitor L-NAME. When cultures were treated with both L-NAME and nicotine, there was a significant reversal of the nicotine-induced myopathy at all concentrations of L-NAME tested; on the other hand, D-NAME the biologically inactive enantiomer had no effect (fig. 4). It should be noted that the culture medium in which the muscle cells were grown contains L-arginine (0.5 mM); for this reason, relatively high concentrations of the reversible NOS inhibitor L-NAME were required. Also, the decrease in the number of branch points in the presence of L-NAME only was significantly different from the control untreated muscle cells (fig. 4), partly explained by the use of high

concentrations of L-NAME. Collectively, the results with NOS inhibitors suggest a role for NO in nicotine-induced degeneration of muscle cells.

2.4.4. Effect of the NO donor SNP on the nicotine-induced myopathy

Because the NOS inhibitors protect against the effects of nicotine, it is conceivable that NO donors may result in degenerative effects on the muscle cultures and/or increase the myopathic effects of nicotine. To test this hypothesis, we exposed the muscle cultures to the spontaneous NO donor, SNP in the absence or presence of 10^{-5} M and 3 x 10^{-5} M nicotine. SNP was also added 4-6 h after the initial exposure to drugs. SNP resulted in a dose dependent decrease in the number of branch points (fig. 5B; fig.6) after a 24 h exposure period as compared to control (fig. 5A). Muscle cultures were then exposed to increasing concentrations of SNP in combination with nicotine (Table 1). Nicotine (10^{-5} M, 3 x 10^{-5} M) resulted in a respective 35 ± 12% and 22 ± 6% decrease in the number of branch points as compared to the control condition (100 ±5%). When muscle cultures were exposed to either 10^{-5} M or 3 x 10^{-5} M nicotine and 3 x 10^{-6} M SNP in combination, the decrease in muscle branch points was similar to that observed with nicotine alone. These present results suggest that SNP mimics the degenerative effects of nicotine possibly by acting through a common pathway or, alternatively through a distinct pathway that results in common morphological manifestations.

2.4.5. Effect of drugs which modify NO levels on ^{125}I-α-BGT binding to muscle cells

To investigate the possibility that the NOS inhibitor NIO and the NO donor SNP exert their effect through a direct interaction at the nicotinic receptor, the effect of these drugs was determined on ^{125}I-α-BGT binding to neonatal muscle cultures. Table 2 shows that neither NIO nor SNP altered specific binding of ^{125}I-α-BGT to the nicotinic receptors.

2.5. DISCUSSION

The present results show that exposure of neonatal muscle cultures to nicotine resulted in a dose dependent decline in myotube branching, which was blocked by the nicotinic receptor antagonist d-tubocurarine. The nicotinic receptor blocker d-tubocurarine on its own (10^{-4} M) neither affected the degree of myotube branching nor the morphology of the muscle cell. These results are in line with previous studies which showed that the administration of acetylcholinesterase inhibitors, which result in an increased level of acetylcholine, produce extensive muscle necrosis through a nicotinic receptor mediated mechanism.

Several reports indicate that activation of the nicotinic receptor at the neuromuscular junction leads to a significant Ca^{++} influx (Decker et al., 1990; Mulle et al., 1992; Vernino et al., 1992). Calcium entry may play a role in the rapid regulation of synaptic function, as well as in long term processes such as agonist induced myopathy (Leonard and Salpeter, 1979, 1982). Subsequent molecular pathways mediating this myopathy have not been identified. However, evidence has shown that NO is involved in calcium mediated cellular toxicity in other systems and that NOS is present in skeletal muscle and is a physiological modulator of skeletal muscle function (Kobzik et al., 1995). Moreover, nNOS is also concentrated at the synaptic junctions of the motor end plates in skeletal muscles (Brenman et al., 1995) colocalizing with both α1-syntrophin, a dystrophin-associated protein, and nicotinic receptors (Brenman et al., 1996). Recently, Brenman et al. (1996) have demonstrated that nNOS is associated with α1-syntrophin and that this association is lost in Duchenne Muscular Dystrophy, suggesting a possible link between nNOS and nicotinic receptors at the neuromuscular junction in muscle pathology.

The present work indicates that NO may also be involved in nicotinic receptor mediated

myopathy. Evidence for this stems from experiments which show that the reversible NOS inhibitor L-NAME and NIO, an irreversible inhibitor of NOS, both resulted in a dose-dependent inhibition of the myopathic effects of nicotine. Thus, inhibition of NOS partially protected muscle cells against the myopathy. These results suggest that nicotinic receptor activation leads to myopathy via the production of NO. This interpretation is further supported by the observation that the NO donor SNP also resulted in a dose-dependent myotube degeneration similar to the nicotinic receptor mediated effect.

The results of the binding experiments indicate that neither the NO donor SNP nor the NOS inhibitor NIO exerted their effects through an interaction at the nicotinic receptor. Thus, NO formation and the subsequent downstream events leading to muscle cell degeneration are secondary to nicotinic receptor activation in cultured muscle cells.

Consistent with our results demonstrating a myopathic effect of nicotine through NO, increased production of this second messenger has been linked to diverse pathophysiological conditions in other systems including septic and cytokine induced circulatory shock (Moncada et al., 1991) and vascular disorders. In addition, NO has been implicated in ischemic brain damage (Nowicki et al., 1991; Rief, 1993; Zhang et al., 1994; Dawson, 1995; Schulz et al.,1995) due to an overstimulation of CNS glutamate receptors. Moreover, more recent studies have shown that inhibitors of nNOS play a neuroprotective role against NMDA, kainic acid and AMPA induced excitotoxicity (Schulz et al., 1995) and that MPTP-induced Parkinsonism in mice was prevented by the nNOS inhibitor 7-nitroindazole (Pzrzedborski et al., 1996). Neurotoxicity was also attenuated in neuronal nitric oxide synthase knockout mice (Schulz et al., 1996).

Activation of guanylate cyclase, which leads to an increase in cGMP levels, is one of the major

pathways through which NO exerts its physiological effects (Radomski et al., 1990a; Moncada et al., 1991; Schmidt et al., 1993; Lowenstein et al., 1994). NO activates the soluble guanylate cyclase by binding to the heme moiety of the enzyme. This results in an increased cGMP concentration which is associated with vasorelaxation (Ignarro and Kadowitz, 1985; Moncada et al., 1991), inhibition of platelet aggregation (MacMicking et al., 1995), cerebral long term potentiation and memory (Dinerman et al., 1994; Linden et al., 1995), modulation of skeletal muscle activity (Kobzik et al., 1994, 1995), and penile erection (Lugg et al., 1995). In addition, the NO/cGMP pathway has been implicated in the regulation of cerebral blood flow, hypoxia and in NMDA receptor mediated excitoxicity (Choi, 1990; Nowicki et al., 1991; Snyder, 1992; Southam and Garthwaite, 1993). In analogy to these other systems, nicotinic acetylcholine receptor activation has been shown to increase cGMP levels in skeletal muscle (Nestler et al., 1978). Furthermore, Briggs (1992) demonstrated that cGMP analogues and NO generators potentiated nicotinic transmission in rat superior cervical sympathetic ganglion.

Other mechanisms proposed for NO neurotoxicity, as well as its bacterial and tumoricidal actions, include intracellular iron loss, inhibition of the nuclear enzyme ribonucleotide reductase, ADP-ribosylation of the Krebs cycle enzyme aconitase (Zhang et al., 1994) and the inhibition of the mitochondrial electron transport chain (Gross and Wolin, 1995; Kobzik et al., 1995), thereby promoting free radical formation. Interaction of NO with oxygen free radicals, such as the superoxide anion, leads to the formation of peroxynitrite, a potent oxidant and neurotoxic agent (Dawson, 1995; Garthwaite and Boulton, 1995; Schulz et al., 1995, 1996). Other proposed mechanisms for NO mediated cell damage include degradation of iron-sulphur centers resulting in the release of Fe^{++} ions and formation of Fe-nitrosyl complexes (Stamler, 1992). Furthermore, NO-induced DNA damage

can also activate the enzyme 3' 5' poly-ADP ribose synthetase, with a subsequent depletion of cellular energy stores to result in cell death (Zhang et al., 1994).

A wide variety of neuromuscular disorders are characterized by muscle cells injury and degeneration, which may be associated with inflammation (Engel et al., 1994). The induction of iNOS, a high output source of NO, is of relevance to the pathophysiology of muscle disorders. iNOS is activated by infiltrating lymphocytes and macrophages in inflammatory associated myopathies (Engel et al., 1994; Dalakas, 1994). Further evidence for the role of iNOS in myopathy stems from the beneficial effects of anti-inflammatory glucocorticoids in treating muscle tissue pathologies (Kaplan et al., 1990). Glucocorticoid inhibition of iNOS expression has been confirmed in many cellular systems such as macrophages, endothelial cells (Radomski et al., 1990a, 1990b) and muscle tissue (Moncada et al., 1991). This observation may explain the effectiveness of glucocorticoid therapy in protecting muscle tissue integrity against inflammatory processes.

To conclude, the present results show that NOS inhibitors prevented the degenerative effects of nicotine on muscle cells, while SNP, a NO donor, resulted in muscle cell degeneration. These findings suggest that overstimulation of the nicotinic receptor may result in pathophysiological processes through an activation of NOS and subsequent production of NO, which in turn activates other cellular processes leading ultimately to muscle cell injury and death.

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TABLE 2.1.

Effect of SNP and nicotine on the number of myotube branch points.

Six days after plating, nicotine and/or SNP were added to the cultures. Addition of SNP was repeated after a period of 4-5 h. The control condition represents dishes with no SNP added. Results are mean ± SEM of 3-5 separate experiments each using 4-5 culture dishes.

Condition	Nicotine	# of Expts.	% control
Control	none	5	100 ± 5
	1×10^{-5} M	3	35 ± 12**
	3×10^{-5} M	5	22 ± 6**
3×10^{-6} M SNP	none	5	66 ± 5*
	1×10^{-5} M	3	22 ± 12**
	3×10^{-5} M	3	8 ± 6**
3×10^{-5} M SNP	none	5	40 ± 5**
	1×10^{-5} M	3	33 ± 12**
	3×10^{-5} M	3	16 ± 6**

Significance of difference from control in the absence of nicotine: ** $P < 0.001$; * $P < 0.05$

TABLE 2.2.

Effect of SNP and NIO on ^{125}I-α-BGT binding to muscle cells in culture

Binding of ^{125}I-BGT (1.8 nM) to muscle cultures was done as described in the Materials and Methods section.The results in experiment A represent the mean ±SEM of 4 culture wells and are representative of 3 experiments. The values in experiment B represent the mean ± SEM of 8 culture wells from 2 separate experiments.

Experiment	Drug	[Conc.]M	Specific binding	
			fmol/well	% control
A	none	---	13.4 ± 0.8	100
	SNP	3×10^{-5} M	12.9 ± 0.6	96
		1×10^{-5} M	12.6 ± 0.4	94
		3×10^{-6} M	12.0 ± 0.5	90
B	none	---	5.2 ± 0.6	100
	NIO	3×10^{-4} M	4.4 ± 0.4	84
		1×10^{-4} M	4.9 ± 0.2	95
		3×10^{-5} M	4.9 ± 0.9	93

FIGURE LEGENDS

Fig. 2.1. Phase contrast photomicrographs of neonatal muscle cells in culture under control conditions and 2 days after nicotine exposure. Cells were plated and maintained in culture for 3 days, after which time the medium was changed to one containing 10 μM cytosar. One day later, cytosar was removed and nicotine or buffer added: (A) control cells; (B) cells exposed to 3×10^{-5} M nicotine. Note the decline in the number of branch points and muscle fiber length after nicotine treatment (B) as compared to control (A). Magnification 100x.

A

B

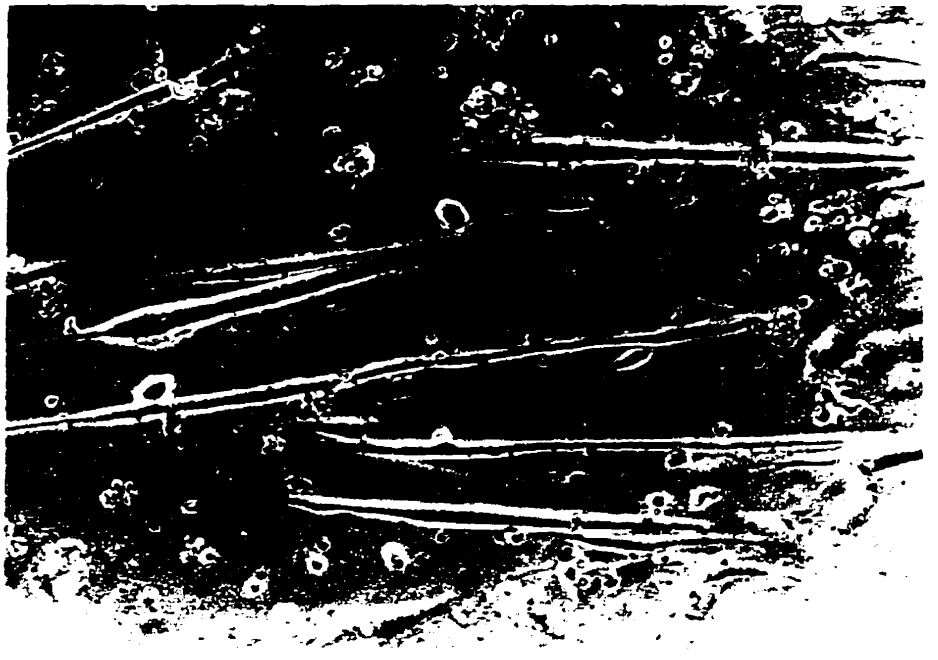


Fig. 2.2. The effect of the nicotinic receptor antagonist d-tubocurarine on the nicotine-induced decrease in myotube branching. Muscle cells were incubated in the absence or presence of d-tubocurarine (10^{-4} M); nicotine/buffer was added 2h later. Changes in cell morphology were assessed after 24h. Nicotine decreased the number of branch points in a dose dependent manner; this was prevented by d-tubocurarine. The results represent the mean ± SEM of 5 culture dishes and are representative of 2 separate experiments. Significance of difference from control in the absence of nicotine: *$P<0.05$, ** $P<0.01$.

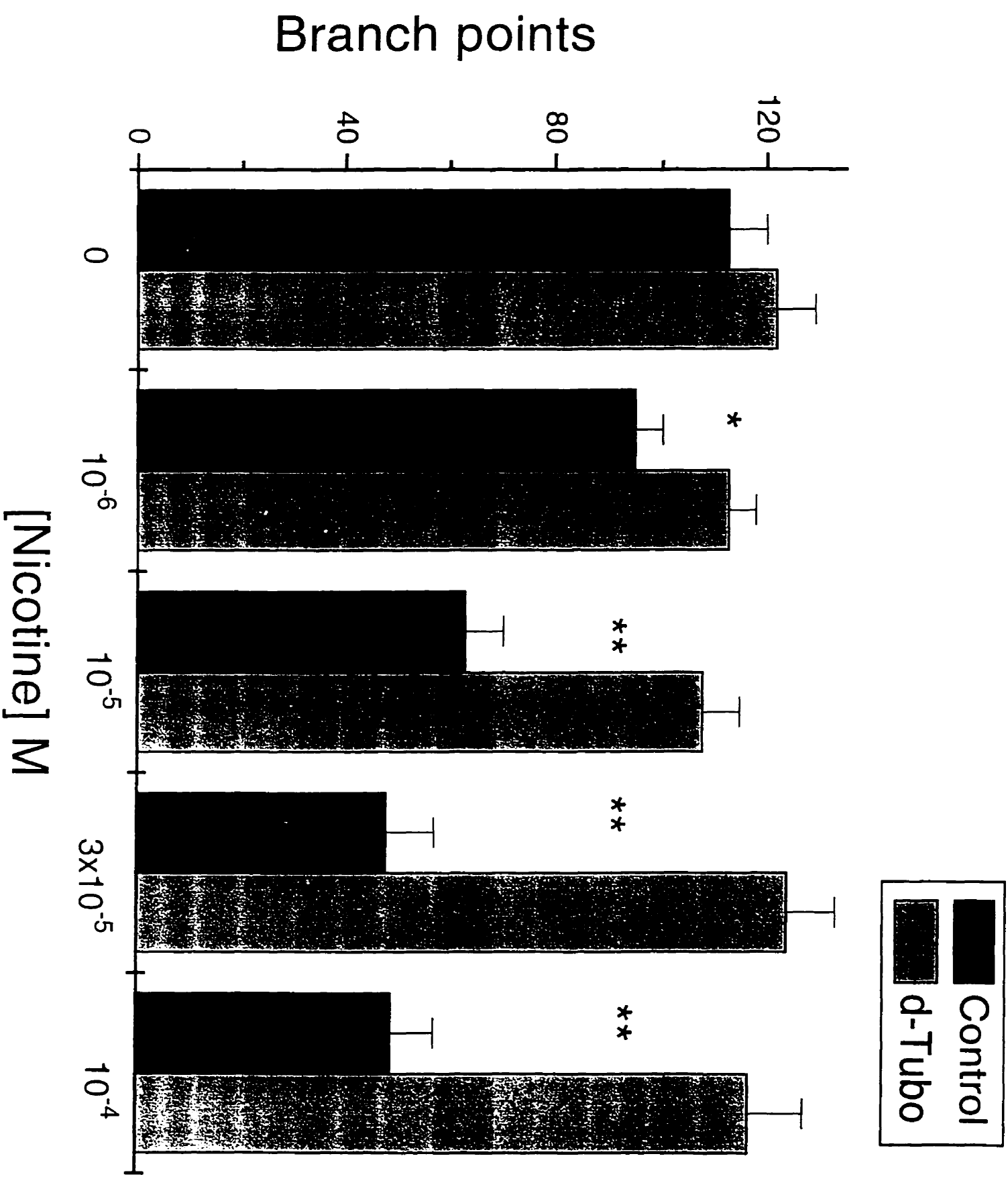

Branch points
0
40
80
120
0
10-6
10-5
3x10-5
10-4
[Nicotine] M
*
**
**
**
Control
d-Tubo

Fig. 2.3. Effect of the irreversible nitric oxide inhibitor NIO on the nicotine induced decrease in muscle branch points. Muscle cells were incubated with different concentrations of nicotine in the absence or presence of 3×10^{-5} M NIO and 10^{-4} M NIO. Note the partial prevention of the degenerative effects of nicotine by different NIO concentrations. Results are the mean ± SEM of 5 culture dishes and are representative of 4 separate experiments. Significance of difference from nicotine in the absence of NIO: *$P<0.05$, **$P<0.01$.

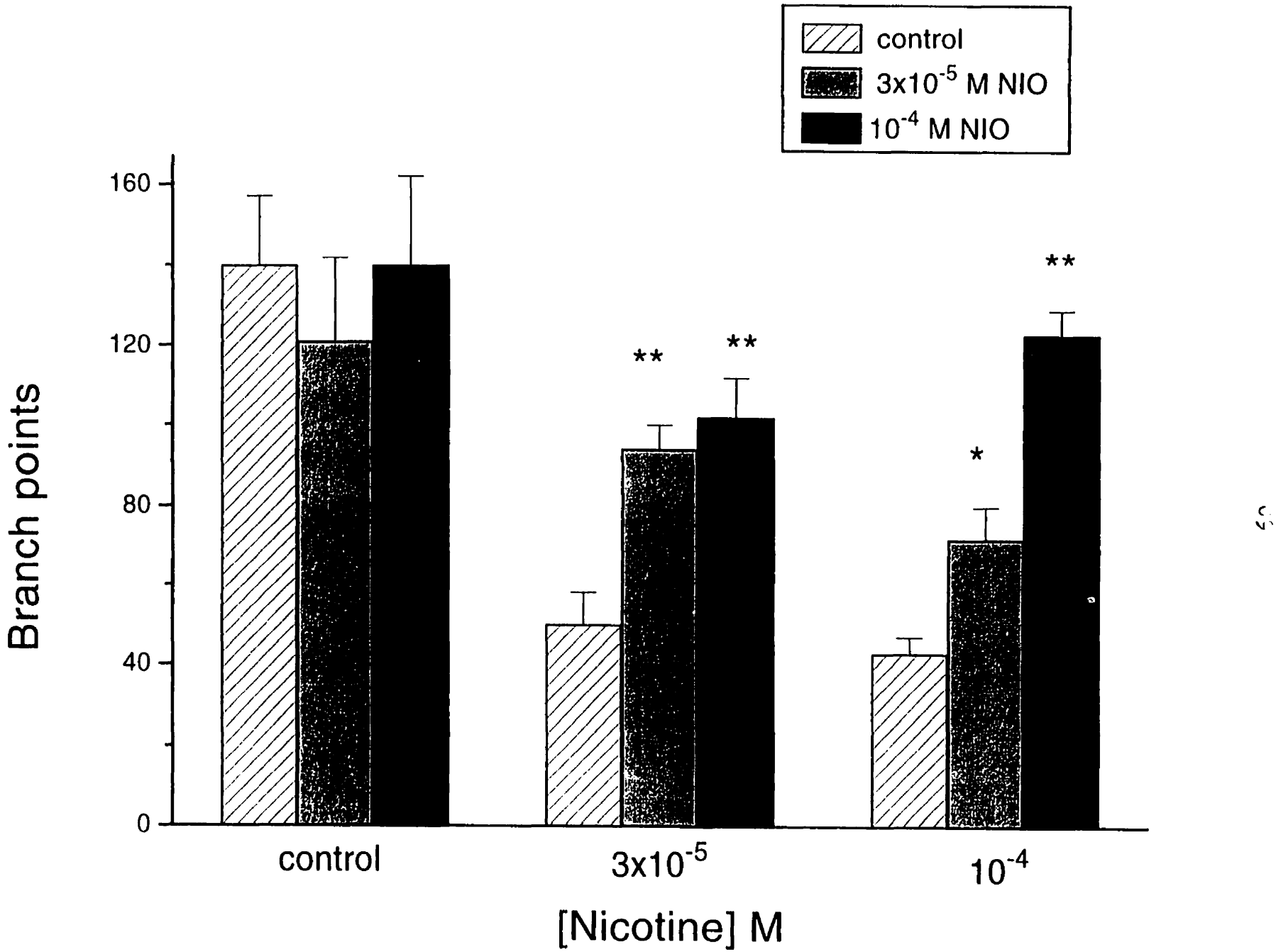
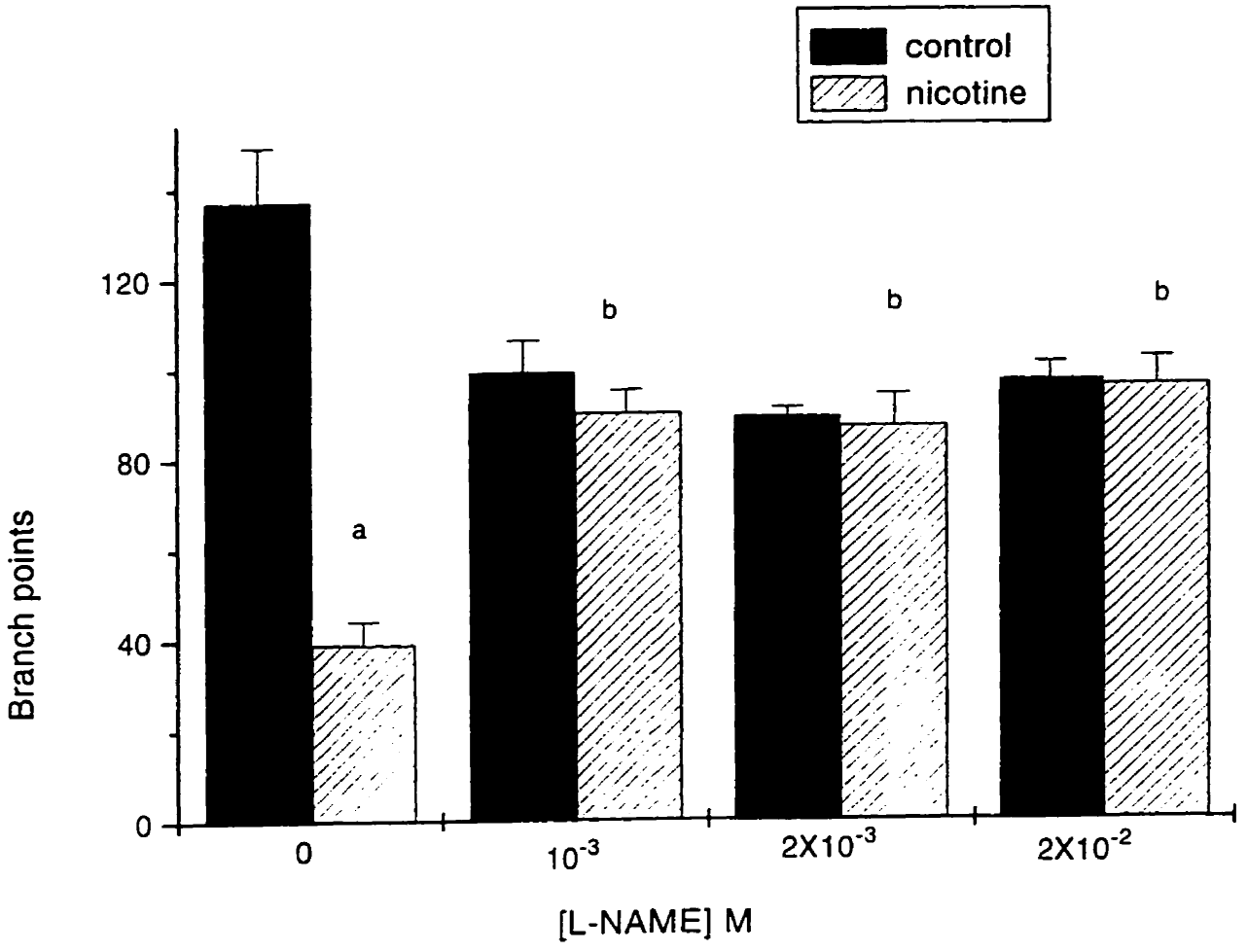

control
3x10-5 M NIO
10-4 M NIO
Branch points
160
120
80
40
0
**
**
**
*
control
3x10-5
10-4
[Nicotine] M

Fig. 2.4.. The effect of the reversible NOS inhibitor L-NAME or the biologically inactive enantiomer D-NAME on the nicotine induced decrease in muscle branch points. Muscle cells in culture were exposed to the indicated concentrations of L-NAME, D-NAME or buffer. Nicotine (3×10^{-5}) or buffer was subsequently added. The number of myotube branch points were counted 24 h later. Note the reversal of the nicotine-induced decrease in the number of branch points at all L-NAME, but not D-NAME, concentrations. The results represent the mean ± SEM of 4 culture dishes and are representative of 4 and 2 experiments for L-NAME and D-NAME, respectively. Significance of difference between control and nicotine treated: $^{a}P<0.001$. Significance of difference between nicotine alone and nicotine in the presence of various concentrations of L-NAME: $^{b}P<0.001$. Significance of difference between control and either nicotine treated only or nicotine treated in the presence of various concentrations of D-NAME: $^{c}P<0.01$.


control
nicotine
Branch points
120
80
40
0
a
b
b
b
0
10-3
2X10-3
2X10-2
[L-NAME] M

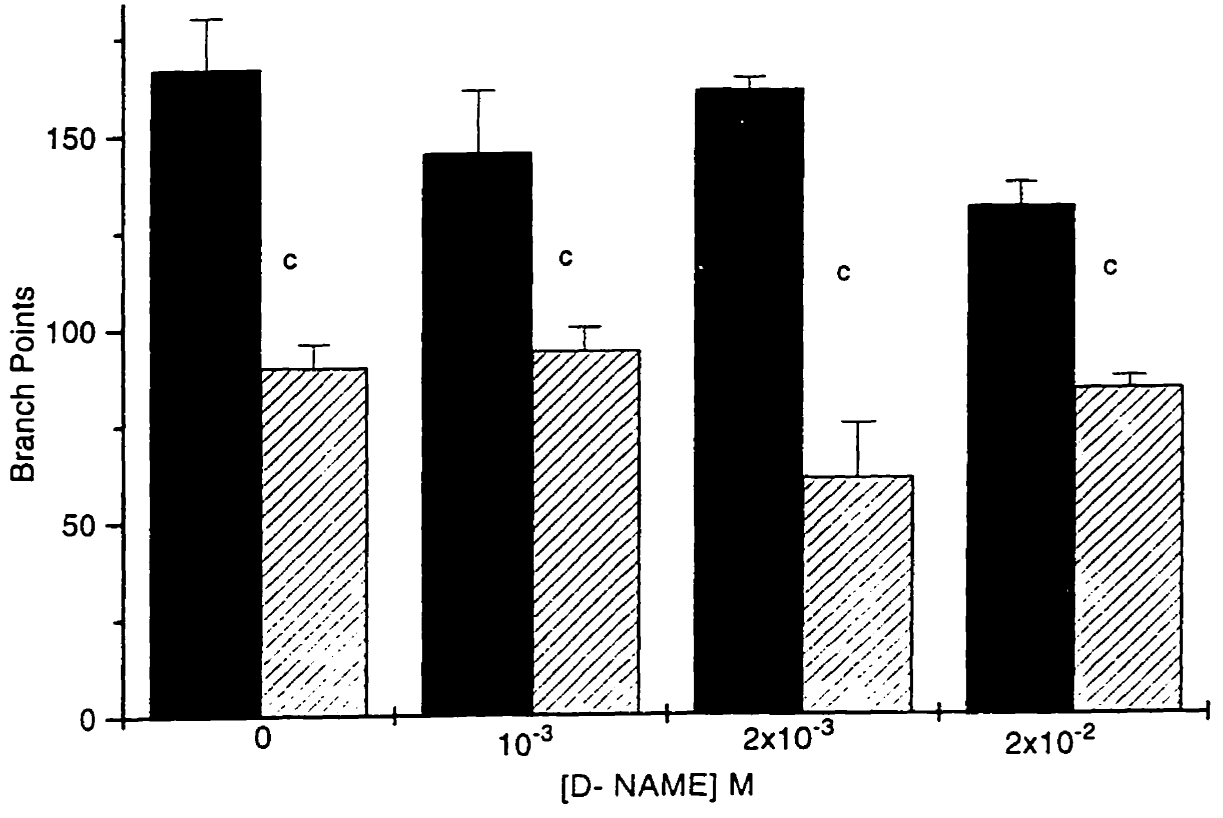
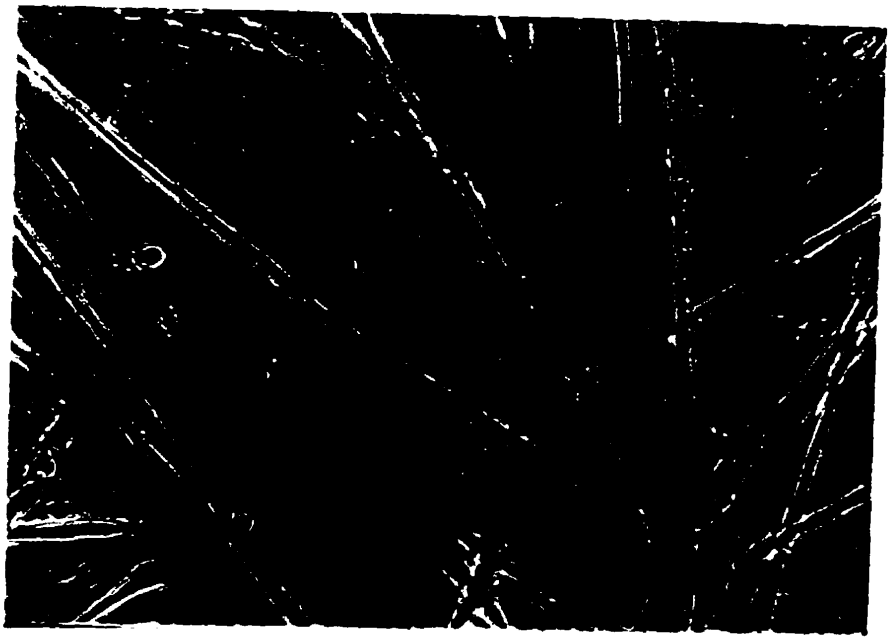

Branch Points
150
100
50
0
c
c
c
c
0
10-3
2x10-3
2x10-2
[D- NAME] M

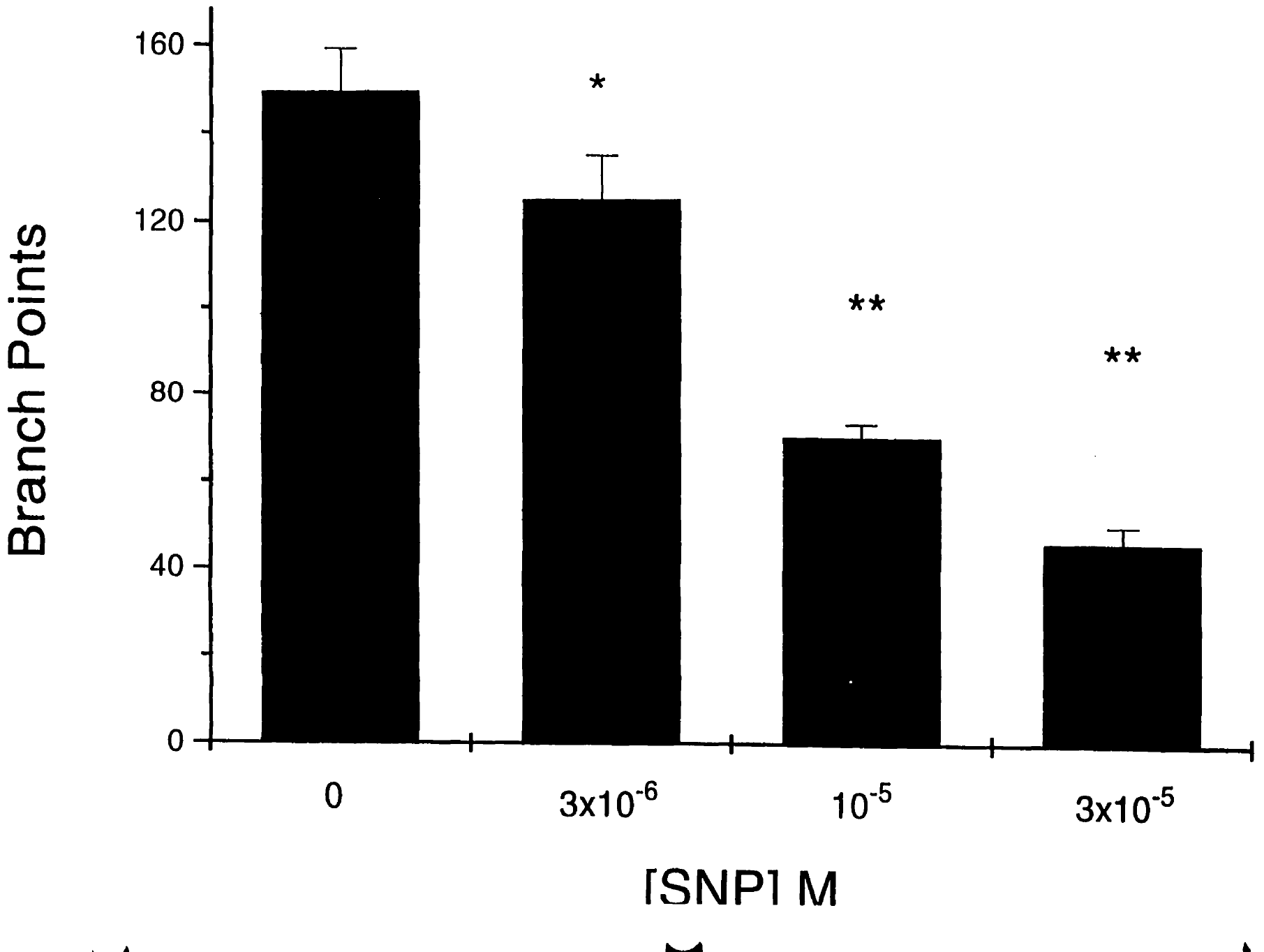
Fig. 2.5. Phase contrast photomicrographs of the effect of the nitric oxide donor SNP on muscle cell morphology. (A) Control cells; (B) cells after a one day exposure to 10^{-5} SNP. Note the reduction in branching after SNP treatment (B) as compared to control (A).

A



B

Fig. 2.6. Effect of SNP on muscle cell branching. Muscle cells were incubated for 2 days in the absence or presence of various concentrations of SNP. Note the dose-dependent decrease in the number of branch points after SNP treatment Results are the mean ± SEM of 5 culture dishes and are representative of 3 separate experiments. Significance of difference between control and SNP treated: *$P < 0.01$; **$P < 0.001$.


Branch Points
160
120
80
40
0
*
**
**
0
3x10-6
10-5
3x10-5
[SNP] M

3.0. The Effect of Nicotine on Nitric Oxide Synthase Activity in Neonatal Skeletal Muscle Cells in Culture

3.1 SUMMARY

Our previous results suggest that changes in nitric oxide synthase (NOS) activity are involved in nicotinic receptor-mediated muscle cell degeneration. To further investigate this possibility, work was done to assess the effect of nicotine on NOS activity in neonatal muscle cells in culture. Experiments were first done to determine whether NOS activity was detectable in neonatal skeletal muscle cells in culture. Enzyme activity was present and exhibited linearity with increasing tissue concentration and time. Studies were then conducted to determine whether nicotine alters NOS activity. The results show that nicotine exposure resulted in an approximate two fold increase in enzyme activity in intact cells in culture. Nicotine had no effect in a lysed muscle cell preparation, suggesting that cellular integrity was essential to produce this effect. These results add further support to the contention that NO may mediate nicotinic receptor induced muscle degeneration.

3.2. INTRODUCTION

Results presented in the previous chapter (El-Dada and Quik, 1997) suggest that NO is involved in nicotinic receptor mediated muscle cell degeneration. We have presented evidence indicating that exposure of neonatal skeletal muscle cell cultures to nicotine results in a dose-dependent decrease in the number of myotube branches. While treatment of muscle cultures with two different NOS inhibitors protected against the degenerative effects of nicotine, the use of sodium nitroprusside (SNP), a NO donor, mimicked the effects of nicotine. Also, α-bungarotoxin binding to nicotinic receptors was not affected in the presence of either SNP or NIO, a NOS inhibitor indicating that the effects of these agents is not through an interaction at the nicotinic receptor.

NO has been assigned to a number of biological functions in a variety of tissues including a prominent role in skeletal muscles activity and development. Immunohistochemical localization of nNOS shows that it is concentrated in the sarcolemma of fast twitch (type II) muscle fibers (Kobzik et al., 1994). Studies using pharmacological modulators of NOS activity suggest that endogenous NO produced in active muscle near the sarcolemma decreases muscle contractile force. Other work has also shown that NO is an important mediator in muscle development and synaptogenesis (Lee et al., 1994; Wang et al., 1995). NO produced by cultured myocytes facilitates myoblast fusion (Lee et al., 1994) and is involved in activity-dependent synaptic suppression in myocyte neuronal cocultures (Wang et al., 1995).

In light of these observations and our previous data, the present experiments were done to further assess the potential involvement of NO in skeletal muscle degeneration. NOS activity was measured using a direct and sensitive technique that monitors the stoichiometric conversion of [^{3}H]-

arginine to [^{3}H]-citrulline. The results provide further support for the hypothesis that NO plays a role in nicotinic receptor induced myopathy.

3.3. MATERIALS AND METHODS

3.3.1. Materials.

Cytosine arabinose (cytosar), bovine serum albumin (BSA), leupeptin, aprotonin, pepstatin, calmodulin, PMSF were purchased from Sigma Chemicals, St. Louis, MO. Calcium chloride ($CaCl_2$), potassium chloride (KCl), sodium chloride (NaCl) and glucose were purchased from Fisher Scientific, Montreal, Quebec. Nicotine hydrogen (+)-tartrate from BDH Ltd., Poole, England. Trypsin, medium 199, minimal essential medium, horse serum, penicillin, streptomycin, ethylenebisoxyethylenenitrilotetraacetic acid (EGTA) and ethylenediaminetetraacetic acid EDTA were purchased from Gibco/BRL, Grand Island, NY. [^{3}H]-arginine, 1 mCi (44.2 Ci / mmol), was purchased from New England Nuclear, Boston, MA. and Dowex 50-WX 8 (sodium form) from Bio-Rad laboratories, Mississauga, Ont.

3.3.2. Muscle cell culture

Myotube cultures from 1-2 day old Sprague-Dawley rats were prepared as previuosly described (El-Dada and Quik, 1997). Minced muscle cells were plated onto 35 mm collagen-coated dishes (Nunc) except that the final density was 1.2 million cells/dish.

3.3.3. Nitric oxide synthase activity

NOS activity was determined by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline (Kobzik et al., 1995). Rat skeletal muscle cultures were plated at a density of 1.2 x 10^6 cells / plate. The plates were washed with 2 x 1 ml HBSS and the cells scrapped off the dish with 2 x 0.5 ml HBSS. The cell suspension was centrifuged for 3 min at 700 - 800 rpm. The cell pellets were then homogenized in 5 volumes of homogenization buffer (50 mM Hepes pH 7.5, 1 mM EDTA, pepstatin 10 µg/ml, leupeptin 10 µg/ml, aprotonin 10 µg/ml, PMSF 100 µg/ml and ß- mercaptoethanol 5 mM). A 25 µl aliquot of the homogenate was added to 25 µl 100 nM [^{3}H]arginine (50 Ci / mmol) and 50 µl of incubation buffer C (50 mM Hepes pH 7.5 containing 20 µg a/ml calmodulin, 4 mM NADPH and 0.45 mM $CaCl_2$, 5 mM KCl, 120 mM NaCl, 10 mM glucose). After incubation for 45 min at 37 °C, the reaction was terminated by addition of 0.5 ml 20 mM Hepes pH 5.5 containing 2 mM EDTA and 2 mM EGTA. The samples were applied to a 1 ml columns of Dowex 50-WX8 (sodium form) and eluted with 2 x 0.5 ml distilled water. [^{3}H] citrulline was quantified by liquid scintillation spectroscopy of the 1.6 ml flow-through. For experiments in which cells were lysed, the incubation buffer I (50 mM Hepes pH 7.5 containing 20 µg/ml calmodulin, 4 mM NADPH and 0.45 mM $CaCl_2$) was used.

3.4. RESULTS

3.4.1. Characterization of NOS activity in neonatal rat skeletal muscle cell cultures

NOS activity was present in skeletal muscle cells and that the activity of the enzyme increased with increasing tissue concentration (fig. 1). To optimize the conditions for maximal NOS activity, experiments were conducted by varying the incubation time and the concentration of

arginine. The incubation time required to obtain a linear increase in NOS activity was first assessed. NOS activity increased with time reaching a maximum at 45 min (fig. 2).

To determine the concentration of arginine needed to produce maximal NOS activity, cells were incubated for 45 min with increasing concentrations of cold arginine and NOS activity measured. The maximal NOS activity was observed with the addition of 10 μM L-arginine; 100 μM L-arginine resulted in enzyme activity more or less similar to that at 1.5 μM L-arginine (table 1).

3.4.2. Effect of nicotine on NOS activity

Cells in culture were exposed to 10^{-4} M nicotine for 30 min. Cells were then scrapped off the dish and incubated for 45 min at 37°C in either (1) an isotonic buffer, buffer C, containing NaCl, KCl, and glucose to preserve the cells in an intact state, or (2) in a hypotonic buffer, buffer I, which contained no KCl, NaCl and glucose thus causing the muscle cell to lyse. Cofactors and [^{3}H]-arginine were added for the incubation period. NOS activity increased with a 30 min exposure to nicotine in intact cells but not in lysed cells (table 2). In a different set of experiments, cells in culture were exposed to nicotine and NOS activity was measured at different time intervals (fig. 3). An increased NOS activity was observed at all time points measured, which was statistically significant from control untreated cells at 15, 30 and 45 min (*$P < 0.001$).

3.5. DISCUSSION

The present results show that NOS activity is present in neonatal skeletal muscle cultures in line with previous studies (Nakane et al., 1993; Kobzik et al, 1994, 1995; Brenman et al., 1995, 1996). The enzyme activity increased with increasing tissue concentration and reached maximal activity after a 45 min incubation at 37°C. Thus, the peak activity of NOS in cultured myoblasts appears to be concentration- and time- dependent. Experiments conducted by other investigators to monitor the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline demonstrated peak NOS activity following a 10 min incubation period at 22°C (Nakane et al., 1993; Lee et al., 1994), 15 min at 22°C (Kobzik at al., 1994), 25 min at 22°C (Brenman et al., 1995) and at intervals ranging from 10-30 min at 37°C (Hobbs et al., 1994; El-Fakahany and Hu, 1995) . These experimental differences could be attributed to factors which include, the type of tissue assayed and the developmental stage. The level of NOS activity in muscle tissue appears to change during the course of myogenic differentiation (Lee et al., 1994).

Our results indicate that the addition of 10 μM L-arginine to the cell cultures prior to incubation produced a marked increase in NOS activity; however, the activity of the enzyme declined to control levels at concentrations higher than 10 μM. One possible explanation could be that, at higher concentrations, arginine inhibits the catalytic activity of the enzyme. Alternatively, the synthesized NO may exist a negative feedback on NOS to modulate its own synthesis has been suggested (Rogers and Ignarro, 1992).

Our study is the first to demonstrate that exposure of skeletal muscle to nicotine increases NOS activity; this might play a role in the agonist induced muscle cell degeneration. These findings indicate that the cytotoxicity induced by nicotine may involve mechanisms similar to the

excitotoxicity induced by the NMDA glutamate type of receptor in the brain. In this regard, excessive activation of NMDA receptors has been associated with a wide range of acute neurologic disorders and chronic neurodegenerative diseases, including hypoxic-ischemic brain injury, trauma, epilepsy, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and acquired immunodeficiency syndrome (Dawson et al., 1993 a, b; Lipton et al., 1993; Dawson et al., 1993 a, b; Bredt and Snyder, 1994; Lipton and Rosenberg, 1994; Gross and Wolin, 1995; Schulz et al., 1995a, b) It is possible that excessive increases in intracellular calcium levels may lead to increased constitutive NOS activity. This in turn may cause an increased release of NO (Garthwaite and Boulton, 1995; Gross and Wolin, 1995), which could damage neurons in the brain (Huang et al., 1994; Strijbos et al., 1996). In a similar fashion, prolonged calcium influx through activated nicotinic receptors has been shown to mediate agonist induced myopathy (Leonard and Salpeter, 1982). Thus, both the neurodegenerative effects mediated by NO in brain and in skeletal muscle might involve a comparable cascade of events relevant to the pathogenesis in muscle tissue.

In conclusion, we have demonstrated that NOS activity is present in rat neonatal skeletal muscle cultures and that it increases with exposure to nicotine. These latter results further substantiate the hypothesis that NO is a mediator of nicotinic receptor induced muscle cell degeneration.

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TABLE 3.1.

Effect of varying arginine concentrations on skeletal muscle NOS activity

NOS activity in neonatal rat skeletal muscle was measured as described in Materials and Methods. The results represent the mean ± SEM of 3 separate experiments each in 5 replicates, except for the results corresponding to 0 μM L-arginine where the results represent only 2 experiments each in 5 replicates.

Arginine (μM)	**NOS activity (pmol/10^6 cells)**
0	10.4, 14.8
1.5	15.9 ± 3.8
3	16.8 ± 2.4
5	18.6 ± 3.8
7.5	16.8 ± 1.1
10	25.5 ± 4.5
100	17.3 ± 2.2

TABLE 3.2.

NOS activity in intact and in lysed skeletal muscle cells

Neonatal rat skeletal muscle cells in culture were exposed to 10^{-4} M nicotine for 30 min. Cells were resuspended for 45 min in either isotonic buffer, or hypotonic buffer, and NOS activity determined as described in Materials and Methods. Data are the mean ± SEM of 5 culture dishes and are derived from 3 separate experiments.

Treatment	Intact cells (Isotonic buffer)	Lysates (Hypotonic buffer)
	NOS activity (pmole/10^6cells)	
Control	38.4 ± 2.6	30.0 ± 1.7
Nicotine (10^{-4} M)	66.6 ± 3.5 *	37.0 ± 2.5

Significantly (*P< 0.001) different from control values.

FIGURE LEGENDS

Fig. 3.1. Measurement of NOS activity with increasing tissue concentration. Neonatal rat muscle culture homogenates were incubated for 45 min as described in Materials and Methods. Results are the mean ± SEM of 3 culture dishes and are derived from 3 separate experiments.

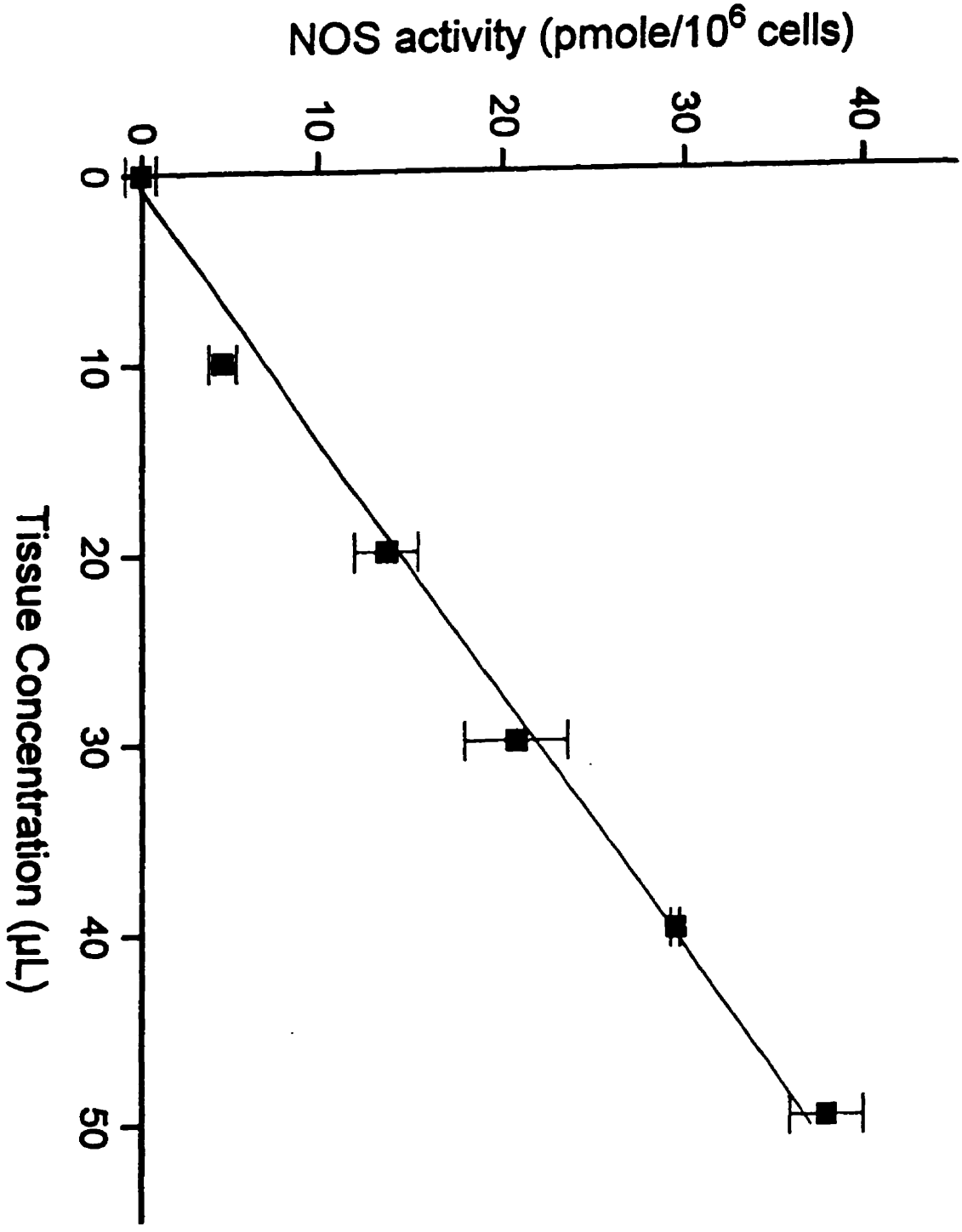

NOS activity (pmole/10^6 cells)
0
10
20
30
40
Tissue Concentration (µL)
0
10
20
30
40
50

Fig. 3.2. Effect of increasing incubation time on NOS activity in cultured rat neonatal skeletal muscle homogenates. Cell homogenates were incubated for the indicated time intervals and NOS activity determined. Note the linear increase in NOS activity with time. Results are the mean ± SEM of 5 culture dishes and are representative of 3 separate experiments.

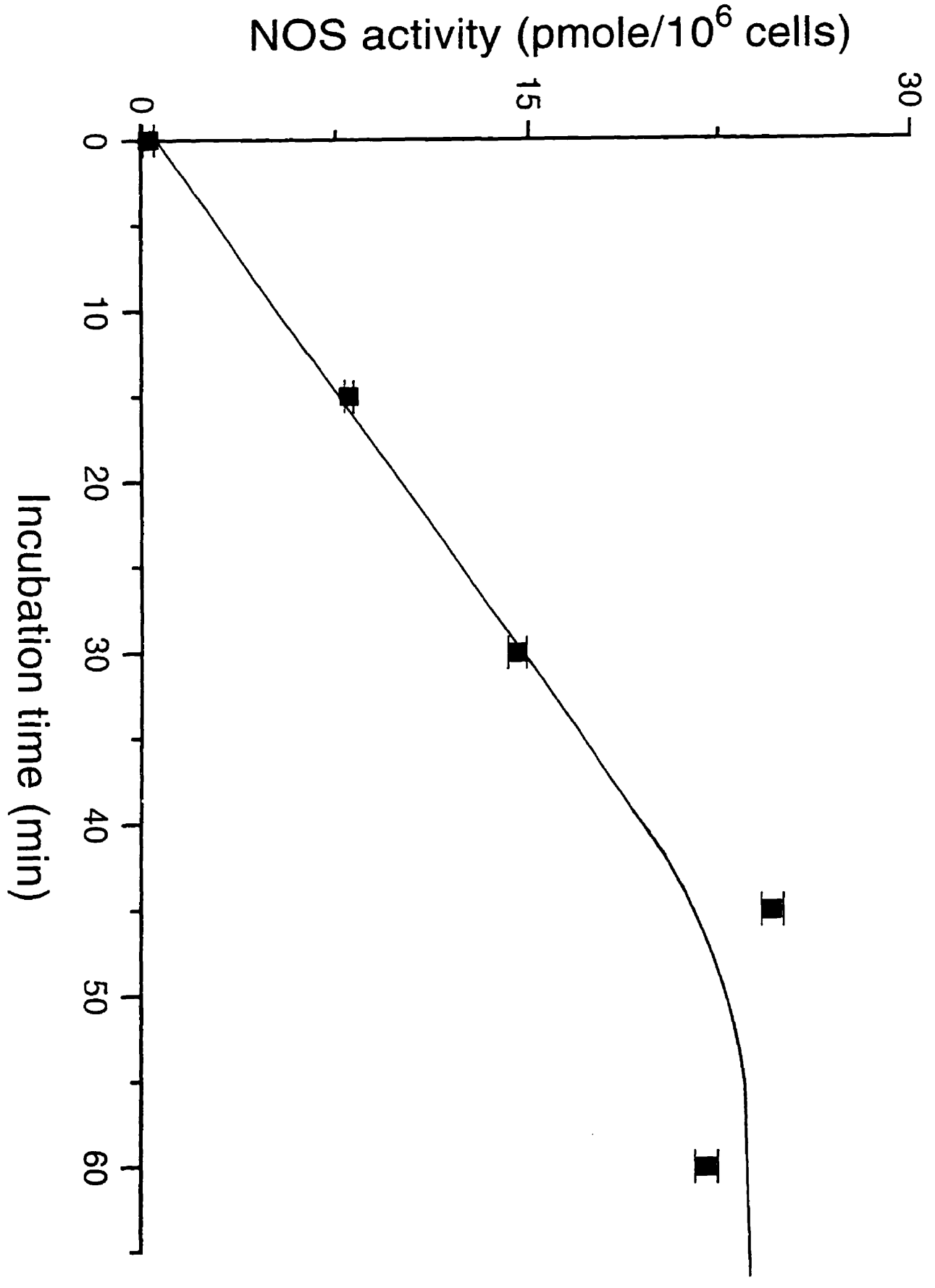
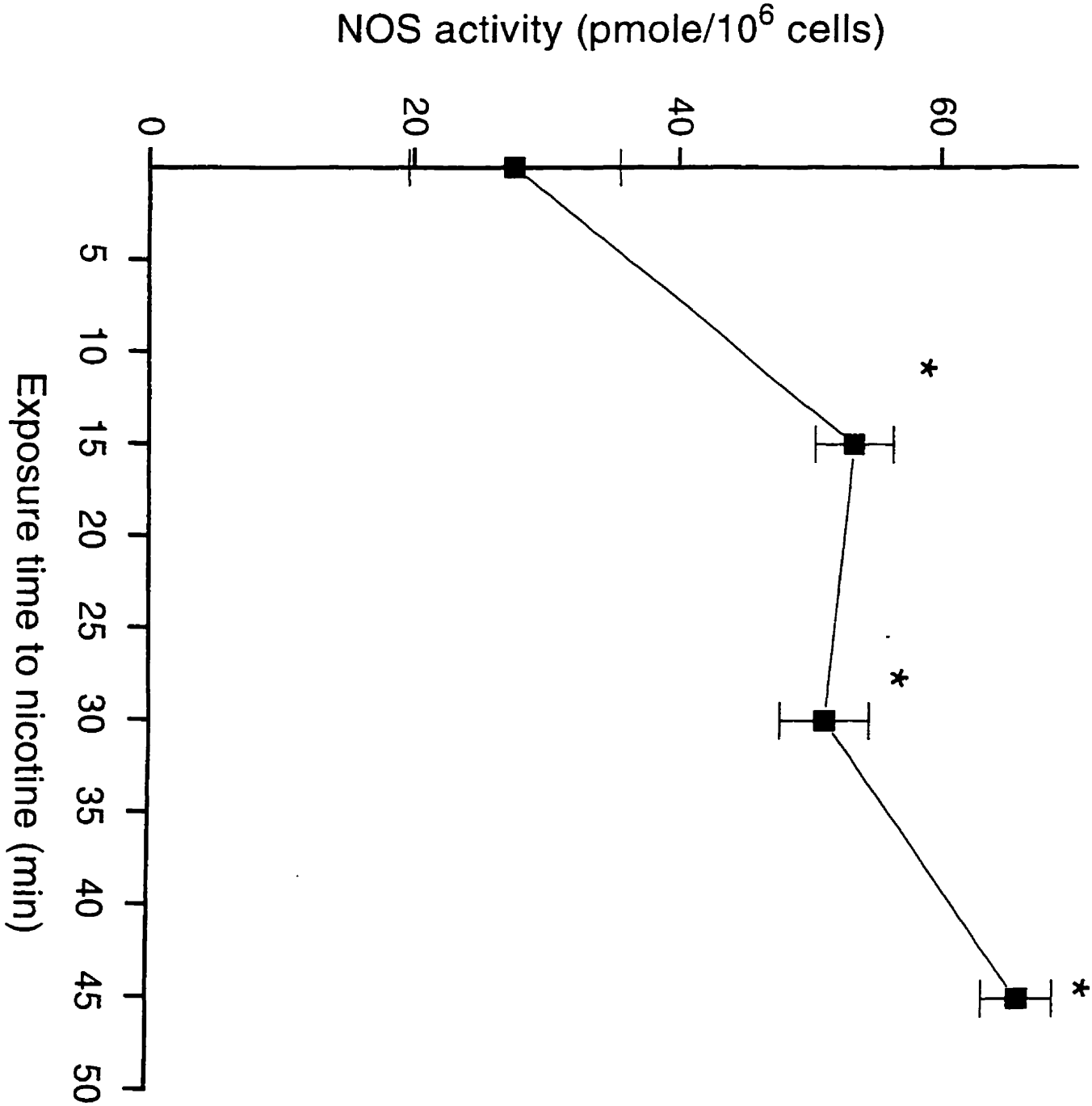

NOS activity (pmole/10^6 cells)
0
15
30
Incubation time (min)
0
10
20
30
40
50
60

Fig.3.3. Effect of nicotine on NOS activity in cultured neonatal rat skeletal muscle cells. Cells in culture were exposed to 10^{-4} M nicotine for the indicated time intervals and NOS activity measured. Results represent mean ± SEM of 4 culture dishes and are derived from of 3 separate experiments. Significantly (*P< 0.001) different from control.


NOS activity (pmole/10^6 cells)
0
20
40
60
Exposure time to nicotine (min)
5
10
15
20
25
30
35
40
45
50
*
*
*

4.0. DISCUSSION

It has previously been reported that agonist induced myopathy at the NMJ could be prevented by administration of nicotinic receptor blockers or by calcium chelators (Fenichel et al., 1972, 1974; Laskowski et al., 1977; Hudson et al., 1978). These observations indicate that nicotinic receptor activation results in a calcium dependent muscle cell. Quantitative measurements of calcium flux indicate that activation of nAChR at the NMJ leads to a significant calcium permeability (Decker and Dani, 1990). Activity dependent calcium influx through the nAChR produces intracellular signals important in synaptic development, maintenance and plasticity. At the NMJ, calcium also modulates nAChR synthesis (Berlin et al., 1990), turnover (Rotzler et al., 1991) and clustering (Bloch and Steinbach, 1981). Calcium is also involved in protein phosphorylation which may directly modulate nAChR activity at the NMJ (Nestler and Greengard, 1984; Berg et al., 1989).

Similar to its role at the NMJ, alteration of calcium levels in the brain have the potential for important regulatory consequences which may as well be detrimental. One of the most prominent examples of this is provided by the NMDA type of glutamate receptors. These receptors are ligand-gated ion channels having a high permeability to calcium, an alteration of which is responsible for processes as diverse as long term potentiation (Garthwaite and Boulton, 1995), elimination of retinotectal projections during development (Wu et al., 1994), and NMDA receptor induced toxicity (Huang et al., 1994; Troy et al., 1995; Dawson and Dawson, 1996). Interestingly, NOS activity was also detected in these activity dependent processes (Huang et al., 1994; Lin and Bennett, 1994; Wu et al., 1994).

A growing amount of evidence implicates NO in skeletal muscle activity, development and dystrophy. NO is formed in skeletal muscle by nNOS which is selectively enriched at the sarcolemma of fast twitch muscle fibers (Kobzik et al., 1994), the fibers preferentially affected in Duchenne muscular dystrophy (DMD) (Webster et al., 1988). Recent studies identify nNOS as a nonstructural component of the dystrophin complex at the NMJ (Brenman et al., 1995; Bredt, 1996). Absence of dystrophin in DMD results in disruption of the dystrophin associated complex and a dramatic decrease in nNOS levels (Brenman et al., 1996). Recently, Chao et al. (1996) have demonstrated that the association of nNOS with dystrophin is also lost in human biopsies of Becker's muscular dystrophy (BMD).

On the basis of these observations, it was hypothesized that there might exist a link between NO and agonist induced myopathy at the NMJ. The present study was undertaken using neonatal rat skeletal muscle cells in culture as a model to extend the previous findings on agonist induced muscle cell degeneration (Fenichel et al., 1972, 1974; Laskowski et al., 1977; Hudson et al., 1978; Leonard and Salpeter, 1979). Exposure of neonatal muscle cultures from 1-day old Sprague-Dawley rats to nicotine resulted in a dose-dependent muscle cell death. We have investigated the effect of nicotine on neonatal skeletal muscle cultures in the absence or presence of NOS inhibitors. Addition of the irreversible NOS inhibitor, L-NIO, or L-NAME, a reversible NOS inhibitor protected the neonatal muscle cells in culture against the degenerative effects of nicotine. These results suggested an involvement of NO in the myopathic effects of nicotine. In contrast to the protective role of NOS inhibitors, incubation of muscle cells with SNP, a NO generating agent, resulted in a dose dependent degeneration of muscle branch points and a subsequent death of the cultured myocytes. When muscle cultures were exposed to both nicotine and SNP, the degenerative effects were similar to that

observed after nicotine alone. These findings further suggest that NO may mediate nicotinic receptor-induced myopathy and that SNP may mimic the degenerative effects of nicotine through a common pathway. Alternatively, NO may result in skeletal muscle cell degeneration via other distinct pathway(s) critical for the normal activity and maintenance of the cell (Dawson and Dawson, 1996). This is supported by previous reports indicating that NO inactivates critical iron-sulfur enzymes including mitochondrial aconitase, nuclear ribonucleotide reductase and succinate:ubiquinone reductase (Drapier and Hibbs, 1986; Lepoivre et al., 1990; Dawson and Dawson, 1996). Also, evidence indicates that NO kills cells by inhibiting the mitochondrial respiratory chain, deenergizing the mitochondria and mobilizing mitochondrial calcium (Cleeter et al., 1994; Richter et al., 1994; Shweizer and Richter, 1994; Brown et al., 1995).

Since NO is implicated in skeletal muscle physiology and structure, it is possible that alterations in the activity of NOS may lead to degenerative events on muscle cells. Endogenous NO plays a role in skeletal muscle contractility, AChR function, myocyte development and activity-dependent synaptic suppression at the NMJ (Kobzik et al. 1994; Lee at al., 1994; Wang et al., 1995). Thus, alteration or disruption of these signalling pathways may lead to abnormal muscle functioning and incomplete myofiber regeneration such as the case in muscular dystrophy (Brenman et al., 1995, 1996; Chao et al., 1996).

An important finding of this study was the marked increase in NOS activity upon exposure of muscle cell cultures to nicotine. While the increase in NOS activity was demonstrated in intact skeletal muscle cells, the same period of 30 min exposure to nicotine had no effect on the activity of the enzyme in lysed skeletal muscle. Additionally, there was a significant increase in muscle cells NOS activity at all time intervals of exposure to nicotine. These results imply that nicotinic receptor

activation results in increased NOS activity in skeletal muscle cells which may in turn mediate a cascade of events that may lead to myopathy. Measurements of NOS activity lend further credence to the results of our previous myotube branch point counts in the presence of either NOS inhibitors or the NO donor, SNP.

NO has previously been shown to be cytostatic for a variety of cell types including skeletal muscle myoblasts (Hess et al., 1993; Peunova and Enikopolov, 1995; Wang et al., 1995; Stangel et al., 1996). Erecinnska et al. (1995) have concluded that NO inhibits oxidative phosphorylation, which in turn decreases ATP levels in synaptosomes and inhibits energy-generating pathways. Additionally, experiments have previously shown that free radical oxygen intermediates (ROI) are present in high levels in skeletal muscle (Davidson et al., 1988; Reid et al., 1992) and may contribute to cytotoxic damage associated in various muscle diseases including DMD (Bredt, 1996). ROI have been implicated as mediators of excitotoxic (Schulz et al., 1995) and apoptotic (Greenlund et al., 1995) neuronal death; they may serve as effectors of cell death, resulting in oxidative damage of DNA, lipids, and proteins (Schulz et al., 1996; Simonian and Coyle, 1996). Stangel and coworkers (1996) have demonstrated that both ROI and NO may also induce apoptosis in myoblasts in a dose-dependent manner. Evidence indicates (Brenman et al., 1996; Chao et al., 1996) that derangement of nNOS in dystrophic muscle might facilitates the interaction of NO with the superoxide radical (O_2^-) generating peroxynitrite ($OONO^-$) a cytotoxic radical which may contribute to muscle necrosis (Radi et al., 1991, 1994; Bredt, 1996).

The participation of NO produced by the cytokine-inducible isoform of NOS, iNOS, in immunological defence mechanisms, has been identified as a cytotoxic factor that can injure normal cells when produced in excess and for an extended period of time (Gross and Wolin, 1995; Strijbos

et al., 1996). Cytokines and endotoxin stimulate the expression of iNOS; NO produced by this isoform functions as both a cytostatic and cytotoxic molecule (McDaniel et al., 1996). iNOS cDNA sequences have been detected to be constitutively expressed at low levels from human skeletal muscle tissues (Williams et al., 1994; Park et al., 1996). Moreover, NO produced by iNOS activates both the constitutive and inducible isoforms of cyclooxygenase to further augment the production of the proinflammatory mediators including prostaglandins, thromboxane and NO (Corbett et al., 1993, 1995). Excess production of NO via this pathway may be important in skeletal muscle cell fatigue, weakness and atrophy.

Immunohistochemical studies have previously revealed the presence of inducible NOS (iNOS) in cultured skeletal muscle cells (Williams et al., 1994) and in skeletal muscle of aged mice with spontaneous myositis (Tamir et al., 1995). Chronic production of NO in combination with oxygen radicals results in DNA damage in addition to tumor necrosis factor (TNF-α)-induced oxidative stress. Further support that NO may play a role in skeletal muscle metabolism was given by a recent observation that treatment of animals with either a NO donor or a superoxide generating system decreased the expression of both myosin (Vale, 1996) and creatinine phosphokinase (MCK). MCK catalyzes the formation of ATP from phosphocreatine and is thus critical for differentiated skeletal muscle function (Wolosker et al., 1996) and energy generation. Together, these results may suggest that NO plays a role in muscle wasting, fatigue and dedifferentiation induced by oxidative stress (Reid et al., 1993; Buck and Chojkier, 1996).

Numerous investigators (Dawson et al., 1991, 1994; Huang et al., 1994; Schulz et al., 1995b) have demonstrated that a marked increase in the levels of NO occurs in the brain during focal cerebral ischemia. Once formed, NO can react with the superoxide anion (O^{-}_{2}), levels of which are

also increased during cerebral ischemia, to form peroxynitrite ($OONO^-$). Peroxynitrite-mediated neuronal injury involves the activation of the nuclear protein, poly(ADP-ribose) synthetase (PARS) (Dawson, 1995; Troy et al., 1996). Consistent with this notion, nNOS knockout mice models were protected from the neurodegenerative effects of NMDA-glutamate receptor overactivity which further confirms that NO may contribute to neurodegeneration (Huang et al, 1994; Dawson and Dawson, 1995; Panahian et al., 1996). Accumulating evidence has recently indicated that prolonged application of nNOS inhibitors may also protect against numerous neurodegenerative processes induced by NMDA receptor overactivity including stroke, Alzheimer's, Huntington's chorea, amyotrophic lateral sclerosis (Lipton et al., 1994; Schulz et al., 1995; Zhang, 1995; Panahian et al., 1996). In more recent experiments, transgenic animals overexpressing superoxide dismutase (SOD) and animals treated with SOD before focal ischemia have a markedly attenuated infarct volume (Estevez et al., 1995; Troy et al., 1996), further indicating the effects of NO and its derived reactive oxygen species (Schulz et al., 1995; Przedborski, 1996) in excitotoxicity. Similar to their role in the brain, NOS inhibitors also protected skeletal muscle cells against ischemia-reperfusion induced injury (Phan et al., 1994) and the severity of inflammatory muscle diseases (Tamir et al., 1995) indicating a role for NO and its derived intermediates, or adducts, in skeletal muscle cell damage.

Taken together, these results suggest that the NO model may provide important new insights into the pathogenesis of both types of muscular dystrophies, DMD (Brenman et al., 1995, 1996) and BMD (Chao et al., 1996). Therefore, the findings presented in this thesis may be of relevance for future studies and concomitant therapies for disorders/myopathies involving nicotinic receptor activation at the NMJ. In a more general sense, our results lend further credence to the protective

role of NOS inhibitors against the myopathy resulting from nAChR overstimulation at the NMJ. However, it will be important to define the events both upstream and downstream of the activation of NOS more precisely, the signalling pathway(s) involved in the myopathy and the effects of prolonged application of NOS inhibitors on skeletal muscle and other tissues where NO has an important modulatory function.

5.0. REFERENCES FOR INTRODUCTION AND GENERAL DISCUSSION

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IMAGE EVALUATION
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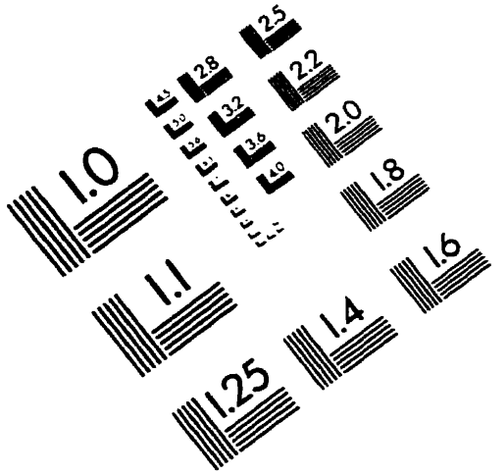

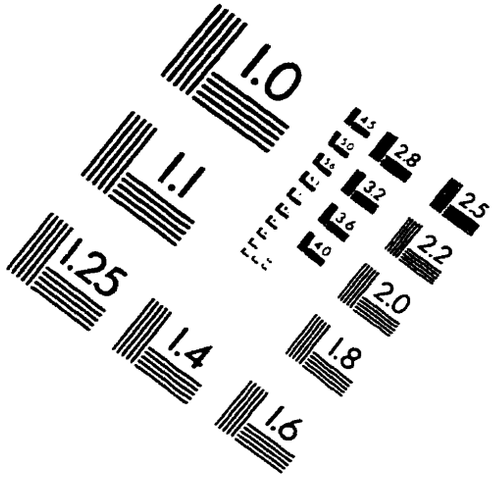

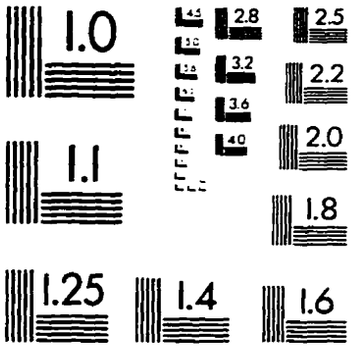

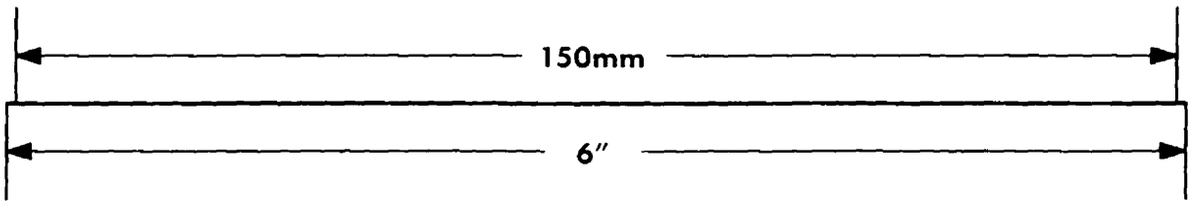

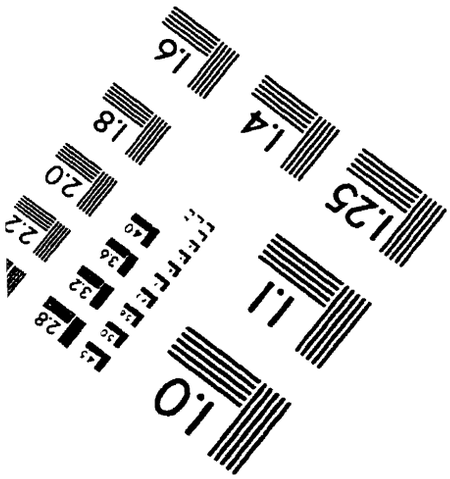

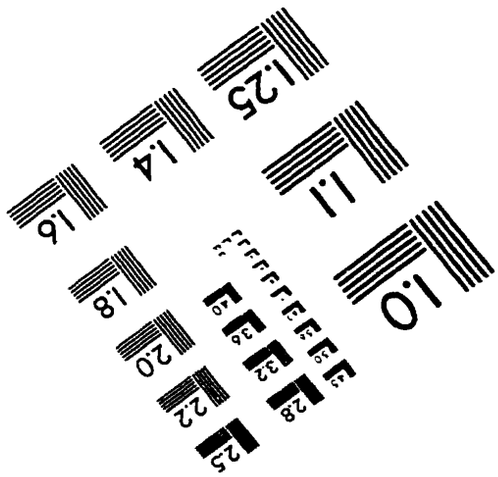

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