Skeletal Muscle and Jejunal Protein Synthesis in Normal and Ethanol-Treated Rats: The Effect of the Nitric Oxide Synthase Inhibitors, L-ω-Nitro-L-Arginine Methyl Ester and N(G)-Nitro-L-Arginine In Vivo

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The nitric oxide synthase (NOS) inhibitors, L-ω-nitro-L-arginine methyl ester (L-NAME; 25 mg/kg and 100 mg/kg) and N(G)-nitro-L-arginine (L-NNA; 100 mg/kg) were used to investigate the role of NO on in vivo skeletal muscle and jejunal (mucosa and seromuscular layer) protein synthesis rates in normal (ie, untreated) and ethanol-dosed (75 mmol/kg body weight) rats. Fractional rates of protein synthesis, ie, percentage of protein pool renewed each day, k_s, %/d) were measured with a flooding dose of L-[^{3}H-4]phenylalanine. In response to both doses of L-NAME and L-NNA, k_s in skeletal muscle of normal rats decreased by 9% to 31% (*P* between < .05 and < .001). In the mucosa, k_s was significantly reduced only by the higher dose of L-NAME (−49%, *P* < .001). In the seromuscular layer, k_s was reduced by 15% to 57% (*P* between < .05 and < .001) in response to both doses of L-NAME and L-NNA. Ethanol dosage reduced k_s in skeletal muscle (−35%, *P* < .001), and small reductions also occurred in the jejunal mucosal and seromuscular layers (−14% *P* < .05 and −12% *P* < .05, respectively). However, in the presence of L-NAME, ethanol reduced k_s in jejunal mucosal and seromuscular layers by −35% (*P* < .01) and −30% (*P* < .01), respectively, compared with controls. This exacerbating effect of L-NAME predosage in ethanol-treated rats was not demonstrable in skeletal muscle. The data thus suggest that (1) endogenous NO facilitates constitutive skeletal muscle and jejunal protein synthesis in control animals in vivo; (2) the sensitivity of jejunal (but not skeletal muscle) protein synthesis to acute ethanol is increased when inhibitors of NOS are used. This tentatively implies that, in response to ethanol, overproduction of NO is not involved in the ethanol-induced reduction of protein synthesis in skeletal muscle or the jejunum.
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AS A CONSEQUENCE of ethanol consumption, numerous pathologic changes arise in most mammalian organs, including the small intestine and skeletal muscle. In the small intestine, this includes partial villus atrophy, smooth muscle damage, and various defects of nutrient absorption and disturbances in intestinal motility. Extensive investigations have been made into the pathogenic mechanisms of the deleterious effects of ethanol on the alimentary tract (for example, Marway et al[1] and Bulut et al[2]). These pathologic changes can be explained, at least in part, by changes in protein metabolism and especially protein synthesis. In selected regions of the small intestine, acute ethanol inhibits mixed protein synthesis by 25% to 30%.[1]

In skeletal muscle, reduced muscle strength occurs due to loss of constituent protein. Although alcohol-induced muscle disease is arguably the most prevalent skeletal muscle disorder, the mechanisms underlying its pathogenesis are poorly understood. Both chronic ethanol abuse and acute misuse, i.e., binge drinking, increase urinary nitrogen excretion with concomitant loss of skeletal muscle protein and thus lean tissue mass.[3] Studies in humans have found rates of skeletal muscle protein synthesis in chronic alcoholics (>100 g/d, >10 years) to be approximately 40% lower than controls.[4]

The gaseous free radical nitric oxide (NO), is an inter- and intracellular messenger involved in a variety of physiologic and pathologic processes, including modulation of vascular tone and neurotransmission. In skeletal muscle, NO is synthesized via neuronal (sarcolemma-associated) and endothelial (mitochondria-associated) isoforms of NO synthase (NOS). In normal skeletal muscle, functions attributed to NO include mediation of neuromuscular transmission, muscle contraction, mitochondrial respiration, and glucose metabolism.[5,6] There are also many sources of NO in the small intestine. The endothelial cells of blood vessels and enteric nerve fibers and varicosities contain NOS and can produce NO. Other sources of NO are interstitial cells in the mysenteric plexus and mucosal macrophages. Thus, NO is constantly produced and is an important neuromodulator of gastrointestinal function. NO regulates muscle tone, mucosal blood flow, and controls peristaltic activities and sphincter function in the gastrointestinal tract.[7,8]

The complex interaction between ethanol and the NO system has been characterized to some extent in various tissues and cell lines. Keshavarzian et al[9] demonstrated that some features of ethanol-induced oesophageal motor changes were mediated through NO pathways as they were either prevented or attenuated by NOS inhibitors. Also, inhibition of NOS exacerbates alcohol-induced gastric mucosal injury.[2] However, the interaction between NO and ethanol has not previously been investigated in either skeletal muscle or the jejunum of the small intestine.

The effects of acute ethanol dosage on the production of NO or its metabolites (nitrate/nitrite) have not previously been reported. It is difficult to investigate directly the role of NO in the regulation of protein synthesis, as measurement of NO production at specific sites is technically difficult in vivo. Under physiologic conditions, NO is formed in small amounts

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and has a very short half-life. Production of NO can be estimated indirectly through the measurement of metabolic products of NO in blood.[10] However, it is uncertain how well nitrate/nitrite levels correlate with NO production. Furthermore, the relative production of NO at specific central or peripheral sites cannot be measured through systemic blood levels of nitrate/nitrite.

The objectives of the present investigation were to determine, using the NOS inhibitors L-ω -nitro-L-arginine methyl ester (L-NAME) and N(G)-nitro-L-arginine (L-NNA): (1) the role of NO in protein synthesis in normal rat skeletal muscle and jejunum in vivo and (2) the effect of NOS inhibition on the ethanol-induced reduction of protein synthesis in vivo.

MATERIALS AND METHODS

Male rats were obtained from Harlan Olac (Bicester, Oxon., UK). General chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK) and Fisher Scientific (UK) (Loughborough, UK).

Treatment of Animals

Male Wistar rats (80 to 100 g) were kept in a temperature-controlled and humidified animal house on a 12-hour light/12-hour dark cycle. Animals were used after 1 week when they weighed approximately 100 to 150 g body weight. All animal facilities and the experimental protocols were approved by the Home Office. Rats were weight ranked and divided into groups of equal mean body weights before use.

The experimental procedure for this study entailed a predose (0.5 mL/100 g body weight; intraperitoneally [IP]) period of 30 minutes, followed by an alcohol-dosing period of 2.5 hours (1 mL/100 g body weight; IP). For predosing, rats were injected with either L-NAME (25 or 100 mg/kg body weight, IP) or L-NNA (100 mg/kg body weight, IP). Controls were injected with an identical volume of 0.15 mol/l NaCl where appropriate. For the dosing period, rats were injected with ethanol at a dose of 75 mmol/kg body weight, IP; controls were injected with identical volumes of 0.15 mol/l NaCl where appropriate. The methods of alcohol dosing have previously been described.[1] The doses of L-NAME (25 mg/kg, IP[11] and 100 mg/kg IP[12]) and L-NNA (100 mg/kg, IP[13]) used in the current study have previously been shown to be effective in rats in vivo. At the dose of 25 mg/kg, IP, L-NAME has been shown to prevent castor oil-induced diarrhea in rats in vivo.[11] At a dose of 100 mg/kg, IP, L-NAME inhibited both incidence and intensity of the audiogenic seizures, which appear in rats at 6 hours postethanol withdrawal.[12] At a dose of 100 mg/kg, IP, L-NNA results in essentially complete neuroprotection after focal ischemia in rats.[13]

Methods for Measuring Protein Synthesis

Ten minutes before the end of the 2.5-hour ethanol treatment period, rats were given a flooding dose of L-[4-^{3}H] phenylalanine (injected at a dose of 15 μmol/100 g body weight) through a lateral tail vein to label the intracellular and extracellular free amino acid pools. After 10 minutes, rats were killed by decapitation, the hind limbs were stripped of skin, and plunged into an ice and water slurry. The gastrocnemius muscles were dissected, blotted, and frozen in liquid nitrogen until processing. The gastrointestinal tract was rapidly dissected out, flushed out with ice cold saline, and both whole jejunal sections (20 cm length) and jejunal seromuscular sections (from 20 cm whole jejunum) were prepared, frozen in liquid nitrogen, and stored at −70°C until processing. The methods of processing the tissues have previously been described.[14]

Calculation of Synthesis Rates

Fractional rates of protein synthesis (defined as the percentage of tissue protein renewed each day [ie, %/d]) was calculated from the formula:

$$k_s = \frac{S_b \times 100}{S_i \times t},$$

where S_b is the specific radioactivity of phenylalanine incorporated into tissue protein (dpm/nmol); S_i is the specific radioactivity of free phenylalanine in acid soluble fractions of tissue homogenates (dpm/nmol); t is the period of time (in days) between injection of the isotope and immersion of tissue in the ice-water slurry.

Statistics

All data are presented as mean ± SEM. Statistical analysis was performed using the pooled estimate of variance, which included analysis of data not presented here, ie, control rats also acted as control groups for other studies not presented here, according to Home Office guidelines to minimize the use of animals. Control data was not historical, but contemporary. Thus, all rats were killed within the same period, and all samples analyzed simultaneously. Significance was assumed when $P \leq .05$; not significant (NS).

RESULTS

Effect of L-NAME on Skeletal Muscle Protein Synthesis

The NOS inhibitor L-NAME (25 mg/kg) reduced k_s in skeletal muscle in vivo, by 20% ($P < .05$). The higher dose of L-NAME (100 mg/kg) also reduced k_s (−31%; $P < .05$) compared with saline controls (k_s, 15%/d). These data are presented in Table 1.

Effect of L-NAME on Jejunal Mucosal and Seromuscular Protein Synthesis

L-NAME (25 mg/kg) reduced k_s in the jejunal seromuscular layer by 15% ($P < .05$) in vivo, but did not affect protein synthesis in the jejunal mucosa. However, a higher dose of L-NAME (100 mg/kg) reduced the rates of protein synthesis in both the jejunal seromuscular layer (−57%; $P < .001$) and the jejunal mucosa (−49%; $P < .001$) compared with saline controls (mucosal k_s, 170%/d; seromuscular layer k_s, 83%/d). These data are presented in Table 2.

Table 1. Effects of L-NAME and L-NNA on Skeletal Muscle Protein Synthesis In Vivo

Group	k_s (%/d)	Difference (%)*	*P* Value
Control	14.9 ± 0.7		
L-NAME (25 mg/kg)	12.0 ± 1.0	−20	<.05
L-NAME (100 mg/kg)	10.3 ± 1.3	−31	<.001
L-NNA (100 mg/kg)	12.0 ± 0.7	−19	<.0001

NOTE. Male Wistar rats were injected IP with either NaCl (control), L-NAME 25 mg/kg, L-NAME 100 mg/kg, or L-NNA 100 mg/kg. Rats were killed 3 hours after injections. All data are mean ± SEM of 5 to 10 observations. Differences between the means were assessed using the pooled estimate of variance. Statistical values pertain to differences from group control.

*Difference as percentage of control.

Table 2. Effects of L-NAME and L-NNA on Jejunal Mucosal and Seromuscular Layer Protein Synthesis In Vivo

Group	k_s (%/d)	Difference (%*)	*P* Value
Mucosa			
Control	169 ± 7		
L-NAME (25 mg/kg)	153 ± 6	−10	NS
L-NAME (100 mg/kg)	87 ± 32	−49	<.001
L-NNA (100 mg/kg)	144 ± 18	−15	NS
Serosa			
Control	83.3 ± 3.1		
L-NAME (25 mg/kg)	70.9 ± 3.3	−15	<.05
L-NAME (100 mg/kg)	36 ± 20	−57	<.001
L-NNA (100 mg/kg)	65 ± 5	−22	<.05

NOTE. For details, see legend to Table 1. Statistical values pertain to differences from group control.

Abbreviation: NS, not significant.

*Difference as percentage of control.

Effect of L-NNA on Protein Synthesis in Skeletal Muscle and in the Jejunum

It is possible that the L-NAME-induced reduction in skeletal and smooth muscle protein synthesis represented a specific and pathologic reaction to L-NAME rather than a general response to NOS inhibitors. This was investigated in additional studies using the NOS inhibitor, L-NNA. It was found that L-NNA (100 mg/kg) also reduced the rate of protein synthesis in skeletal muscle by 19% ($P < .0001$; Table 1).

In the jejunal seromuscular layer, L-NNA (100 mg/kg) reduced the rate of protein synthesis by 22% ($P < .05$). However, in the jejunal mucosa, the effect of L-NNA (100 mg/kg) on protein synthesis did not quite achieve significance ($P = .08$). These data (Table 2) suggest that the reductions in protein synthesis in skeletal muscle and the jejunal seromuscular layer observed in response to both L-NAME and L-NNA represent a specific reaction to NOS inhibitors in general.

Effect of Ethanol and L-NAME on Skeletal Muscle and Jejunal Protein Synthesis

Ethanol dosage reduced skeletal muscle k_s (−35% $P < .001$; Fig 1) and also jejunal mucosal and seromuscular layer k_s (−14% $P < .05$ and −12% $P < .05$, respectively, Figs 2 and 3).

In both the jejunal mucosal and seromuscular layers, dosage with L-NAME before ethanol resulted in greater reduction of k_s than administration of either L-NAME or ethanol alone. These results are shown in Fig 2 and 3. However, the ethanol-induced reduction of k_s in skeletal muscle was not affected by predosage with L-NAME (Fig 1).

The seromuscular layer S_i was reduced 9% ($P < .05$) in those animals treated with ethanol alone, and the mucosal S_i was increased 27% ($P < .001$) in animals treated with L-NAME (100 mg/kg). All other effects on skeletal muscle, jejunal mucosal, and seromuscular layer k_s were not due to changes in precursor enrichment, ie, S_i was not altered (data not shown).

DISCUSSION

The role of NO in pathologic intestinal and muscle tissues has paradoxically been reported to be both deleterious and beneficial. Thus, NO has been implicated as a potentially damaging tissue factor after strenuous exercise[15] and is absent in muscle fibers from patients with Duchenne muscular dystrophy.[16] In contrast, there is evidence that NO may protect skeletal muscle tissue after ischemic reperfusion injury in rats.[17] Inhibition of NOS exacerbates alcohol-induced gastric mucosal injury.[2]

NO has been implicated in the regulation of protein synthesis in various tissues, including the liver and vascular smooth muscle.[18] However, the role of NO in protein synthesis in the small intestine has not previously been investigated, and only one previous study has investigated the role of NO in protein synthesis in skeletal muscle. Thus, Fryburg[19] addressed the issue of whether the metabolic actions of insulin-like growth factor-I (IGF-I) could be altered by the use of an NOS inhibitor. In human skeletal muscle, IGF-I exerts both growth hormone-like (increased protein synthesis) and insulin-like (decreased protein degradation and increased glucose uptake) actions. However, when the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) was coinfused with IGF-I for 6 hours into forearm muscle, no significant increase in muscle protein synthesis was

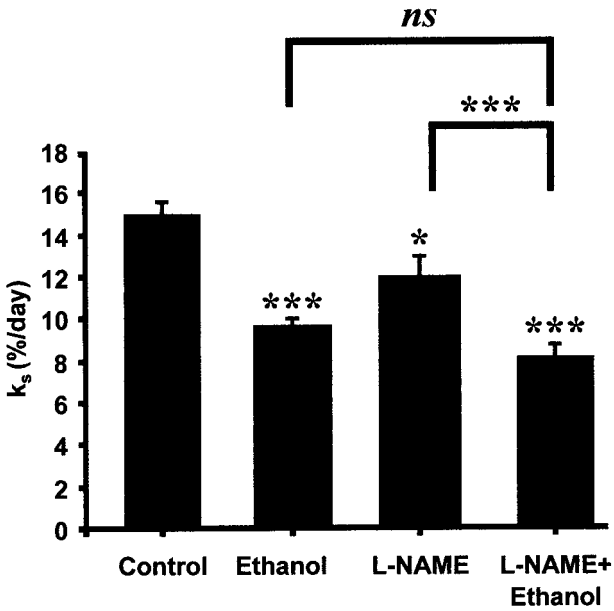

Fig 1. The effects of ethanol and L-NAME on skeletal muscle protein synthesis in vivo. Male Wistar rats were injected IP with either saline (0.15 mol/L NaCl; control), ethanol (75 mmol/kg body weight), L-NAME (25 mg/kg), or L-NAME (25 mg/kg) and ethanol (75 mol/kg body weight). Rats were killed 3 hours after the first injections. All data are mean ± SEM of 5 to 10 observations. Differences between the means were assessed using the pooled estimate of variance. Statistical values pertain to differences from group control. Other differences were as follows: L-NAME *v* L-NAME + ethanol, −32% (*P* < .001). ethanol *v* L-NAME + ethanol, −16% (NS, *P* > .05; **P* < .05; ***P* < .01; ****P* < .001).

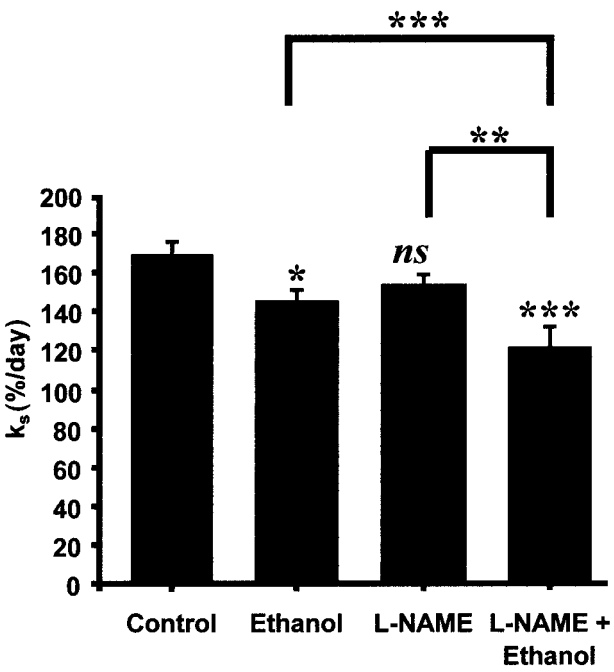

Fig 2. The effects of ethanol and L-NAME on jejunal mucosal protein synthesis in vivo. For details, see legend to Fig 1.

detected, although the other expected metabolic effects of IGF-I were observed.[19]

The data presented from the current study demonstrate that NOS inhibitors reduce protein synthesis in skeletal muscle and in the jejunum in vivo. This suggests that NO may play a positive role in facilitating protein synthesis in these tissues in vivo. This has been suggested in skeletal muscle,[19] but never before in the jejunum. Furthermore, although administration of L-NAME (100 mg/kg) reduced the rate of protein synthesis in both the jejunal mucosa and the seromuscular layer, L-NAME (25 mg/kg) inhibited protein synthesis in the seromuscular layer, but did not affect mucosal protein synthesis. These observations demonstrate that the different layers of the jejunum exhibit varying sensitivities to inhibition of NOS and further suggest that protein synthesis may be differentially regulated in different layers of the jejunum. This has not been demonstrated previously, but is expected given that luminal mucosal cells are continually shed and replaced by the process of cell turnover, while the turnover of cells in the seromuscular layer is much slower.

The mechanisms underlying the inhibitory effects of NOS inhibitors on protein synthesis are unclear at present. In vitro, NO has been reported to inhibit protein synthesis in eukaryotic cells by increasing the phosphorylation of the alpha-subunit of eukaryotic initiation factor (eIF) 2.[20,21] It is possible that modulation of the NO system in vivo has some, as yet, uncharacterized actions on these factors, which initiate protein synthesis. The effects of NOS inhibitors may depend on the reactivity of NO with functional groups and metals in proteins and/or enzymes, including cytochrome P-450, NOS, and heme-regulated eIF 2A kinase.[21-25] Additionally, inhibition of NO synthesis using substrate-based inhibitors of NOS (such as L-NAME) may result in the generation of free radicals, such as superoxide from uncoupled NOS activity.[26]

It is possible that the reduction of protein synthesis observed in the presence of NOS inhibitors could represent alterations of blood flow. Unfortunately, it was not possible to measure blood flow in the current study. To measure blood flow, the rats must be anesthetized, which can result in severe respiratory depression or death in ethanol-treated rats[27] and can also affect NO production.[28-30] However, phenylalanine uptake, as reflected by S_i, was not significantly reduced in those animals treated with NO inhibitors compared with control animals (data not shown for brevity). This suggests that blood flow was not sufficiently reduced as to overtly affect concentrations of the precursor amino acids in either skeletal muscle or the jejunum.

In accordance with previous studies[1,3] protein synthesis in both skeletal muscle and in the jejunum was inhibited by acute ethanol administration. Furthermore, in the current study, treatment with L-NAME (25 mg/kg) before ethanol resulted in greater inhibition of protein synthesis than administration of either agent alone in both the mucosal and seromuscular layers of the jejunum. However, L-NAME (25 mg/kg), did not modulate the ethanol-induced reduction of skeletal muscle protein synthesis.

Protein synthesis in normal jejunal mucosa was inhibited by L-NAME (100 mg/kg), but not at a dose of 25 mg/kg. However, predosage with L-NAME (25 mg/kg) increased the sensitivity of the jejunal mucosa to acute ethanol administration, resulting in greater inhibition of protein synthesis than administration of either agent alone. Protein synthesis in the jejunal seromuscular layer was found to be more sensitive to acute ethanol after predosage with L-NAME. The normal cellular

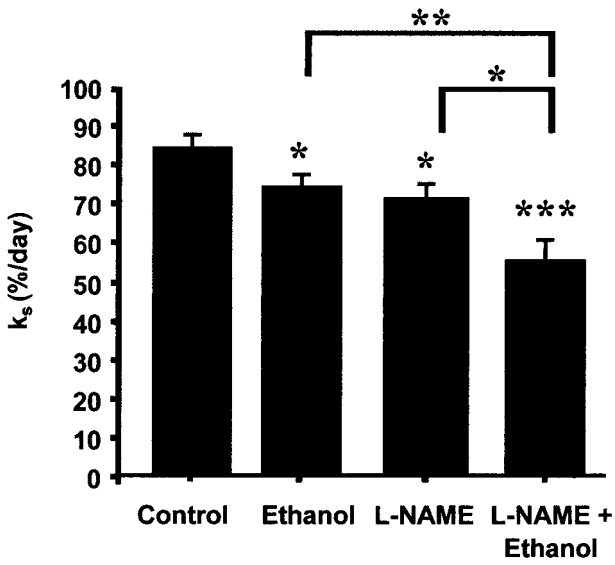

Fig 3. The effects of ethanol and L-NAME on jejunal seromuscular protein synthesis in vivo. For details, see legend to Fig 1.

responses to acute ethanol treatment may therefore be blunted by reduction of NO production. Recently, acute ethanol administration has been shown to affect the protein synthesis initiation factors eIF2B and eIF4E in the liver and skeletal muscle, respectively.[31]

The exacerbation of ethanol's effects on k_s resulting from pretreatment with L-NAME could be explained by a negative effect of NOS inhibitors on the eIF, as postulated above. Alternatively, the increased ethanol toxicity could be related to effects of L-NAME on either blood flow[21-24] or production of free radicals.[25]

In conclusion, the observations of the current study suggest that inhibitors of NOS cannot ameliorate the ethanol-induced reduction of protein synthesis in either skeletal muscle or the jejunum. Other mechanisms may be involved in alcohol toxicity in these tissues, for example, free radical damage,[3,30] although, inhibition of the NO system may actually exacerbate the ethanol-induced reduction of protein synthesis, at least in the jejunum. These observations also demonstrate that the different layers of the jejunum exhibit varying sensitivities to inhibition of NOS. Furthermore, as a result of NOS inhibition by L-NAME, the sensitivity of jejunal mucosal and seromuscular layer protein synthesis to acute ethanol administration was increased.

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