Timing of administration of dexamethasone or the nitric oxide synthase inhibitor, nitro-L-arginine methyl ester, is critical for effective treatment of ischaemia-reperfusion injury to rat skeletal muscle

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INTRODUCTION

Ischaemia-reperfusion (IR) injury to skeletal muscle may occur following tourniquet application, trauma, myocutaneous tissue transfer or replantation of amputated parts in reconstructive surgery. The resulting necrosis compromises the recovery of function of the affected body part. Oxygen-derived free radicals, lipid hydroperoxides, nitric oxide (NO), neutrophil infiltration, cytokines and inflammatory eicosanoids have been implicated in the pathophysiology of this necrosis [1-4]. More recently it has been suggested that oxidatively modified immunoglobulin and activated complement contribute to the acute oedema in IR injury [5].

NO, synthesized from L-arginine by the enzyme NO synthase (NOS), has an important physiological role in the maintenance of vasodilatation and an anti-thrombotic endothelium [6]. In non-inflamed tissues, the Ca2+-dependent endothelial isoform of NOS (eNOS) is the predominant, if not exclusive, source of NO. However, inflammation following IR injury has been shown to lead to the induction of the Ca2+-independent (inducible) form of NOS (iNOS) [7]. NO has a paradoxical profile of biological activity with both cytotoxic and cytoprotective effects [8]. Nitrovasodilators release NO as the nitrosourea ion (NO+) which is considered to be cytoprotective [9]. However, NO has cytotoxic effects per se and as a consequence of its interaction with superoxide anion to form the potent oxidant, peroxynitrite [10]. We have previously shown that NOS inhibition reduces IR injury to skeletal muscle [11] and skin [12], indicating that in these tissues the net effect of NO production is deleterious [4]. A similar conclusion has been reached in a study

Key words: glucocorticoid, ischaemia-reperfusion, nitric oxide, nitric oxide synthase inhibitors, skeletal muscle.

Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible (Ca2+-independent) nitric oxide synthase; i.p., intraperitoneal; IR, ischaemia-reperfusion; l-NAME, nitro-L-arginine methyl ester; l-NIO, nitroiminoethyl-L-ornithine; MPO, myeloperoxidase; 7NI, 7-nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; RT, reverse transcriptase; SMT, S-methylisothiourea.

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examining the effects of NOS inhibitors on acute oedema responses to IR injury in the rat hind limb [3].

In this study we have sought to confirm the cytoprotective effects of arginine analogue NOS inhibitors [13] in IR injury to rat skeletal muscle. The presence of iNOS in reperfused ischaemic muscle has been established by a radiochemical assay and reverse transcriptase (RT)-PCR. In order to assess the clinical potential of NOS inhibition for the treatment of IR injury we have examined the optimal timing of administration of the NOS inhibitor, N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME) and the anti-inflammatory glucocorticoid, dexamethasone.

METHODS

All procedures were carried out with the prior approval of the St Vincent's Hospital Animal Experimentation Ethics Committee and conformed to National Health and Medical Research Council (Australia) guidelines on animal experimentation.

Male Sprague–Dawley rats (250–300 g) were anaesthetized using intraperitoneal (i.p.) pentobarbitone sodium (60 mg/kg), and ischaemia was produced by placing a number 31 elastic band with seven turns to form a tourniquet of 2–3 mm in width as high as possible on the right hind limb of the rat proximal to the anterior tibialis and gastrocnemius muscles. Infusion of India ink into the infrarenal aorta following application of the tourniquet in initial studies confirmed complete cessation of blood flow. The tourniquet was released after between 1.5 h and 2.5 h in experiments to determine an appropriate duration of ischaemia. The criterion for an appropriate level of injury was muscle survival after 24 h of reperfusion. An ischaemia time of 2 h was used in all subsequent experiments. A needle thermistor was placed subcutaneously and proximally to muscle to provide a continuous measure of muscle temperature. During the ischaemic period, the rats were kept in a box and the ischaemic muscle temperature was maintained at 36±1°C with an adjustable lamp. The upper body part of the animals was shielded from the light source to prevent hyperthermia. At the end of the ischaemic period the rats were allowed to recover from anaesthesia and the limb was reperfused for a further 24 h. Control and treated muscles were harvested under anaesthesia using pentobarbitone sodium (60 mg/kg, i.p.) and the animal was killed by administration of 325 mg/kg pentobarbitone sodium after harvest of the muscles.

Drug Administration

In the first series of experiments, the following agents (Sigma Chemical Co., St Louis, MO, U.S.A.), except nitroiminoethyl-L-ornithine (L-NIO) which was obtained from IDT, Boronia, Victoria, Australia, were administered intraperitoneally 30 min before the end of the ischaemia period: saline (1 ml/kg); L-NAME (30 mg/kg); L-NIO, (30 mg/kg); S-methylisothiourea (SMT, 10 mg/kg); 7-nitroindazole (7Ni, 10 mg/kg); dexamethasone (2.5 mg/kg). In another series of experiments, rats were treated by i.p. injection of either saline (1 ml/kg), L-NAME (30 mg/kg) or dexamethasone (2.5 mg/kg), either 30 min before tourniquet application, 30 min before release of tourniquet, at both these aforementioned times, or at 3, 8 or 16 h after the release of the tourniquet.

Assessment of muscle viability

After 24 h of reperfusion, the gastrocnemius muscles were dissected, sliced perpendicular to the longitudinal axis at 2 mm intervals, and incubated for 20 min at room temperature in a phosphate-buffered solution of Nitro Blue Tetrazolium (NBT) dye and NADH [14]. These stained muscle sections were fixed in buffered formal saline and analysed by quantitative image analysis (Video Pro 32, Faulding Imaging, Clayton, Victoria, Australia). The sections were placed under a CCD camera and the non-magnified video image was imported into the image analysis software to detect the blue-staining parts of the muscle (viable muscle fibres) and the total cross-sectional area. These semi-automated analyses were carried out by an operator blinded to the drug treatments of the specimens. The percentage of each muscle slice that was viable was measured by analysing the percentage of the total surface area (both sides of the muscle slice) that stained positively. The weight of the slice was also recorded. The percentage of viable muscle was then determined by multiplying the average percentage area of viable muscle of each slice by the weight of that slice. The sum of these values was divided by the total weight of the whole muscle to provide the final reported percentage viability.

Neutrophil accumulation in 24 h reperfused muscles

Tissue myeloperoxidase (MPO) activity was measured as an index of neutrophil accumulation in reperfused ischaemic skeletal muscles. Specimens were harvested, quickly frozen in liquid nitrogen, freeze-dried and stored at −70°C until the assays were performed. MPO activity was determined by a modification of the method of Suzuki et al. [15]. Briefly, muscle samples (15–25 mg dry weight) were homogenized in 2 ml of 20 mmol/l potassium phosphate buffer (pH 7.4) containing 0.1 mmol/l EDTA using an Ultra Turrax tissue homogenizer. Muscle homogenate (2 ml) was centrifuged at 1500 g for 10 min at 4°C to pellet the insoluble cellular debris. The supernatant, which contains <5% of the total MPO and >95% of water-soluble haemoproteins, was discarded [16]. The pellet was then rehomog-
enzined in 0.05 mol/l potassium phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide. The samples were rapidly freeze–thawed twice in liquid nitrogen/37°C water bath and sonicated in a 37°C water bath for 20 s. MPO activity was assessed by measuring the hydrogen peroxide-dependent oxidation of \( N,N,N',N'\)-tetramethylbenzidine at 37°C for 4 min. The incubation was stopped by adding 0.1 mmol/l catalase and 1.75 ml of cold 0.2 mol/l acetic acid [17]. One unit of enzyme activity was defined as the amount of MPO that caused a change in absorbance of 1.0 per min at 655 nm and 37°C.

Change in muscle water content after 24 h of reperfusion

Oedema formation induced by IR injury was examined in the gastrocnemius muscles which were weighed immediately after freezing in liquid nitrogen (wet weight) and again after a minimum of 24 h of freeze-drying (dry weight). The data are presented as a ratio of wet to dry weight.

NOS activity

Cross-sections of gastrocnemius muscles were immediately frozen in liquid nitrogen then freeze-dried and stored at -70°C until assayed. NOS activity was measured as the conversion of \[^{3}H\]l-arginine (Amersham International, Amersham, U.K.) into \[^{3}H\]l-citrulline by cell-free homogenates [18]. The tissue samples were homogenized on ice in an Ultra-Turrax T 25 homogenizer in a buffer containing 50 mmol/l Tris/HCl, 0.1 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 1 mM PMSF, 10 \( \mu \)g/ml leupeptin, 5 \( \mu \)g/ml chymostatin, 10 \( \mu \)g/ml soy bean trypsin inhibitor, 2 \( \mu \)g/ml apro tin and 320 mmol/l sucrose adjusted to pH 7.4. The buffer was added to tissue in a ratio of 1.6 per ml per 100 mg of tissue dry weight. The homogenate was then centrifuged at 4°C, 10000 g (Sorvall, RT6000D) for 15 min. The supernatant was carefully removed and stored on ice for no more than 1 h before the reaction was started. Supernatant (100 \( \mu l \)) (containing approximately 500 \( \mu \)g protein) was added to 200 \( \mu l \) of a buffer consisting of 1 mol/l dithiothreitol, 10 \( \mu l \) l-arginine, 1 mmol/l NADPH, 100 mmol/l calmodulin, 5 mmol/l tetrahydrobiopterin, 2 mmol/l CaCl\(_2\), 50 mmol/l Hepes, 60 mmol/l l-valine, 1.2 mmol/l l-citrulline, 1 mmol/l l-ornithine and \[^{3}H\]l-arginine (approximately 4 \( \times \)10\(^{4}\) d.p.m.) in 20 mmol/l Hepes buffer adjusted to pH 7.5. Samples were incubated for 60 min at 37°C before the reaction was stopped by addition of 1.5 ml of Hepes buffer (2 mmol/l l-EGTA/2 mmol/l EGTA, pH 5.5). The admixture was applied to 1 ml of Dowex 50W (Na\(^{+}\) form) column which was eluted with 1.5 ml of distilled water. The tritium content of the sample effluent and 1.5 ml of water wash containing the \[^{3}H\]l-citrulline was measured by scintillation counting (LKB scintillation spectrometer). The total NOS activity was determined by measuring the difference in \[^{3}H\]l-citrulline produced between homogenates incubated in the presence and absence of 300 \( \mu \)mol/l l-NO, a concentration which completely inhibits NOS under these cell-free conditions. The \(Ca^{2+}\)-dependent NOS activity was determined as the difference between the total NOS activity and the activity in samples incubated in a reaction mixture containing 2 mmol/l EGTA and no CaCl\(_2\). The \(Ca^{2+}\)-independent enzyme activity, usually equated with iNOS activity in such assays [19], was calculated as the difference between the total and the \(Ca^{2+}\)-dependent NOS activity. All activities are expressed as pmol min\(^{-1}\) (mg of protein\(^{-1}\)). Protein concentration was measured spectrophotometrically using the Bio-Rad reagent (Bio-Read, Hercules, CA, U.S.A.) with BSA as the standard.

Expression of iNOS mRNA in skeletal muscle

iNOS oligonucleotide primers were based on the published rat liver iNOS nucleotide sequence [20]. The forward primer, F1, corresponds to nucleotides 87–106 (beginning 104 bp upstream of the start codon), and the reverse primer, R2, to nucleotides 656–675 (amino acids 153–159; IEEHLAR) within the protein coding region. The primer sequences are: F1, 5'-TAGAGACCCTTCTGGATTTC-3' and R2, 5'-CTGGCCACATGTTCTCTAT-3'. The expected PCR product is 588 bp and is specific for iNOS. \(\beta\)-Actin was used as a control for RNA integrity and amount in each RT-PCR reaction. \(\beta\)-Actin was derived from the rat \(\beta\)-actin nucleotide sequence [21]. The forward primer (corresponding to mRNA nucleotides 199–220), 5'-CTGAGAG-TACCCCATGGAACATGGC-3' and reverse primer (corresponding to mRNA nucleotides 960–937), 5'-CAGAGCAGTAATCTCCTCTTC-3' generates a 762 bp cDNA PCR product.

Muscle specimens were harvested from rats subjected to 2 h of ischaemia and 8 h of reperfusion, frozen in liquid nitrogen and stored at -70°C before RNA extraction. Total RNA was isolated from rat gastrocnemius muscles by guanidinium isothiocyanate and phenol–chloroform extraction [22]. Total RNA (0.5 \( \mu \)g) from rat gastrocnemius muscle was used as a template for cDNA synthesis, using Moloney murine leukemia virus RT and specific iNOS and \(\beta\)-actin primers as described in the GeneAmp RT-PCR kit (Perkin-Elmer Cetus, U.S.A.). Samples incubated without RT or without RNA in the cDNA synthesis reaction served as controls for DNA contamination. iNOS and \(\beta\)-actin RNA were amplified by PCR according to the manufacturer's instructions with 0.5 \( \mu \)g of each iNOS primer and 0.4 \( \mu \)g of each \(\beta\)-actin primer. Amplification was performed on a Perkin-Elmer 2400 PCR machine (Perkin-Elmer Cetus, U.S.A.) in
the following buffer: 10 mmol/l Tris/HCl (pH 8.3), 2 mmol/l MgCl₂, 50 mmol/l KCl, 200 μmol/l of each dNTP, 0.01% (w/v) gelatin and 2.5 units of Taq polymerase (Perkin-Elmer Cetus, USA.). The reaction mix was heated to 94°C for 3.5 min, and 40 cycles of denaturation (94°C, 30 s), annealing (55°C, 40 s) and extension (72°C; 45 s for iNOS, 60 s for β-actin) were carried out. One tenth of the total PCR reaction mixture was resolved on a 1.8% agarose slab gel and the PCR products visualized by ethidium bromide staining. In Fig. 1 the two PCR solutions (iNOS and β-actin) were loaded into the same lane for ease of comparison between samples.

Statistical analyses

Data are presented as the means and SEMs of n observations. Groups of data were analysed by one-way analysis of variance and the significance of individual comparisons were assessed by Newman–Keuls multiple comparison test. A difference was considered to be statistically significant when \( P < 0.05 \).

RESULTS

Influence of duration of ischaemia on subsequent necrosis

In preliminary experiments, ischaemia times of 1.5, 2 and 2.5 h were evaluated, with the reperfusion time remaining constant at 24 h. The percentage viability of skeletal muscle under these conditions was 46 ± 4, 17 ± 2 and 0 respectively (n = 8 in each group). Muscle from sham-operated rats or the contralateral, non-ischaemic hind limb had no detectable areas of infarction (viability 100%). In subsequent experiments, a period of 2 h of ischaemia and 24 h of reperfusion was used to induce significant damage to enable the detection of protective agents.

Effects of NOS inhibitors on ischaemia-reperfusion-induced necrosis of skeletal muscle

Administration of the NOS inhibitors, l-NAME (30 mg/kg), l-NIO (30 mg/kg), SMT (10 mg/kg) or the anti-inflammatory glucocorticoid, dexamethasone (2.5 mg/kg), 30 min before the start of reperfusion, significantly increased the survival of muscle observed in saline-treated animals \( (P < 0.05) \) (Table 1). The neuronal NOS-selective inhibitor, 7NI [23] had no significant effect on muscle survival. In saline-treated rats subjected to 2 h of ischaemia and 24 h of reperfusion, there were significant \( (P < 0.05) \) increases in MPO activity and wet/dry weight ratios of anterior tibialis muscle, and the level of reduced glutathione decreased to approximately 30% of that in sham-operated muscle (Table 1). The protection afforded by the NOS inhibitors was not associated with marked effects on MPO activity or on wet/dry weight ratios, indices of neutrophil influx and tissue oedema respectively (Table 1). Only l-NAME and dexamethasone significantly reduced MPO activity by approximately 15%. Each of the agents which decreased the post-ischaemic necrosis also reduced the tissue wet/dry weight ratio \( (P < 0.05) \) by approximately 25–30%. The depletion of muscle glutathione levels was attenuated by SMT or dexamethasone, but was unaffected by the other agents (Table 1). Further investigation of the effect of l-NAME (1–30 mg/kg, i.p., 30 min before reperfusion) on the level of injury indicated that it produced a dose-related increase in muscle survival, with a maximum effect being achieved at 10 mg/kg (Table 2).
Table 1. Effects of NOS inhibitors on survival, MPO activity, wet/dry weight ratios and glutathione levels in muscle subjected to 2 h of ischaemia and 24 h of reperfusion. All results given ± SEM, with values of n given in parentheses. *P<0.05; ND, not done.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skeletal muscle viability (%)</th>
<th>MPO (absorbance min⁻¹ mg⁻¹)</th>
<th>Wet/dry weight (ratio)</th>
<th>Glutathione (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>100</td>
<td>0.16±0.21 (11)</td>
<td>3.17±0.34 (9)</td>
<td>4.53±0.38 (11)</td>
</tr>
<tr>
<td>Saline</td>
<td>23±4 (15)</td>
<td>8.28±0.20 (10)</td>
<td>5.98±0.26 (10)</td>
<td>1.42±0.05 (11)</td>
</tr>
<tr>
<td>L-NIO</td>
<td>42±3* (10)</td>
<td>7.45±0.23 (7)</td>
<td>5.38±0.06* (8)</td>
<td>1.97±0.14 (9)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>55±3* (10)</td>
<td>7.07±0.33* (6)</td>
<td>5.38±0.23* (9)</td>
<td>1.89±0.22 (8)</td>
</tr>
<tr>
<td>SMT</td>
<td>69±6* (9)</td>
<td>7.28±0.38 (8)</td>
<td>5.19±0.16* (9)</td>
<td>2.47±0.44* (9)</td>
</tr>
<tr>
<td>7NI</td>
<td>28±3 (8)</td>
<td>8.54±0.29 (7)</td>
<td>ND</td>
<td>1.69±0.17 (6)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>53±3* (10)</td>
<td>7.09±0.17* (8)</td>
<td>5.20±0.20* (9)</td>
<td>2.63±0.21* (7)</td>
</tr>
</tbody>
</table>

NOS activity and expression in muscle subjected to IR injury

NOS activity was measured in cell-free homogenates of gastrocnemius muscle by following the conversion of [3H]-arginine into [3H]-citrulline. After 8 h of reperfusion, Ca²⁺-independent NOS levels increased, whereas Ca²⁺-dependent NOS levels had decreased compared with the levels detected in sham-operated rats. Treatment of rats with dexamethasone (2.5 mg/kg) 30 min before reperfusion attenuated the increase in Ca²⁺-independent NOS activity (P<0.05), but had no significant effect on the decrease in Ca²⁺-dependent NOS activity (Table 3). Additional evidence for the presence of iNOS in reperfused muscle was sought using RT-PCR for iNOS expression. Contralateral, non-ischaemic muscle had no detectable iNOS expression, whereas there was detectable expression of iNOS in muscle subjected to 2 h of ischaemia and reperfused for 8 h (Fig. 1). In rats treated with dexamethasone (2.5 mg/kg), the iNOS message was undetectable by RT-PCR in three out of three experiments.

Table 2. Dose-dependence of the effect of L-NAME on survival of skeletal muscle following 2 h of ischaemia and 24 h of reperfusion. Results given as means ± SEM; n = 4.

<table>
<thead>
<tr>
<th>L-NAME (mg/kg, i.p.)</th>
<th>Skeletal muscle viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (1 ml/kg)</td>
<td>16±2</td>
</tr>
<tr>
<td>(no L-NAME)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22±2</td>
</tr>
<tr>
<td>3</td>
<td>34±2</td>
</tr>
<tr>
<td>10</td>
<td>65±5</td>
</tr>
<tr>
<td>30</td>
<td>53±4</td>
</tr>
</tbody>
</table>

Influence of timing of administration of L-NAME or dexamethasone on necrosis

The effects of L-NAME and dexamethasone at the same doses which reduced necrosis when administered 30 min before reperfusion, were evaluated at different times after reperfusion. L-NAME produced protective effects of a similar magnitude when administered after 3 h of reperfusion, but had no effect when administered after 8 h of reperfusion, whereas dexamethasone treatment at either 3 or 8 h after reperfusion showed a similar degree of protection to that achieved with its earlier administration (Table 4).

Neither L-NAME nor dexamethasone improved viability when administered 30 min before ischaemia or after 16 h of reperfusion (Table 4). In a further series of experiments, dexamethasone (2.5 mg/kg) was administered 30 min before ischaemia and either L-NAME or dexamethasone (2.5 mg/kg) was administered 30 min before reperfusion. The pre-ischaemic administration of dexamethasone followed by a further dose of dexamethasone 30 min before reperfusion resulted in a viability of only 13±3% (n = 8). The pre-ischaemic administration of dexamethasone also prevented the protective effects of

Table 3. NOS activities in cell-free homogenates of rat skeletal muscle. Results are means ± SEM, with values of n given in parentheses. *P<0.05, comparison of activities in ischaemic-reperfused and sham-operated tissue. †P<0.05, comparison of activities between saline and dexamethasone-treated ischaemic-reperfused tissue.

<table>
<thead>
<tr>
<th>Muscle treatment</th>
<th>Drug treatment</th>
<th>NOS activity (pmol/min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca²⁺-dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca²⁺-independent</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>Nil</td>
<td>1.20±0.40* (4)</td>
</tr>
<tr>
<td>2 h ischaemia</td>
<td>Saline</td>
<td>0.23±0.10* (10)</td>
</tr>
<tr>
<td>8 h reperfusion</td>
<td>Dexamethasone (2.5 mg/kg)</td>
<td>0.32±0.09* (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.09±0.05† (10)</td>
</tr>
</tbody>
</table>
Timing of drug administration

<table>
<thead>
<tr>
<th>Timing of drug administration</th>
<th>Saline (control)</th>
<th>L-NAME (30 mg/kg)</th>
<th>Dexamethasone (2.5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min before ischaemia</td>
<td>22 ± 9 (8)†</td>
<td>19 ± 4 (8)†</td>
<td></td>
</tr>
<tr>
<td>30 min before reperfusion</td>
<td>14 ± 3 (7)</td>
<td>57 ± 4 (6)*</td>
<td>77 ± 7 (5)*</td>
</tr>
<tr>
<td>After 3 h reperfusion</td>
<td>74 ± 6 (6)</td>
<td>69 ± 3 (5)</td>
<td></td>
</tr>
<tr>
<td>After 8 h reperfusion</td>
<td>7 ± 2 (5)†</td>
<td>76 ± 9 (6)</td>
<td></td>
</tr>
<tr>
<td>After 16 h reperfusion</td>
<td>16 ± 5 (5)†</td>
<td>38 ± 13 (6)†</td>
<td></td>
</tr>
</tbody>
</table>

L-NAME (30 mg/kg) administered before reperfusion (viability 10 ± 3%, *n = 6*). Surprisingly, L-NAME (30 mg/kg) administered 30 min before ischaemia was lethal in four out of four rats subsequently treated with dexamethasone (2.5 mg/kg) 30 min before reperfusion. In addition, four out of six rats died before the end of the 24 h reperfusion period when treated with L-NAME (30 mg/kg) 30 min before ischaemia and a second dose of L-NAME (30 mg/kg) 30 min before reperfusion.

DISCUSSION

The present study provides further evidence for a net deleterious effect of NO in IR injury to skeletal muscle [3, 11]. The findings also indicate that iNOS is produced during the reperfusion of ischaemic muscle, but it remains unclear as to whether NO from iNOS, eNOS or both isoforms contributes to the ensuing injury. The timing of administration of L-NAME or dexamethasone appears to be critical to the protective effect: pre-ischaemic administration of these agents is deleterious, whereas both agents remain effective when administered up to 3 h after reperfusion.

L-NAME is a well-established inhibitor of all NOS isoforms, but shows some degree of selectivity for eNOS [23, 24]. L-NIO is non-selective [13] and SMT is reportedly selective for iNOS [25], although effects on eNOS could not be excluded at the dose used in our study. Because there is no circulation to the affected limb during the ischaemic period, the drug treatments would not reach the muscle until after reperfusion and would then require time to equilibrate with the tissue. For this reason, we cannot exclude a beneficial effect of NO production during the first few minutes of reperfusion. Nevertheless, the similar protective effects of each of the NOS inhibitors is consistent with a net deleterious effect of endogenous NO in IR injury to skeletal muscle at some stage during the first 3 h of reperfusion, as we [11] and others [3] have previously suggested. In contrast, dexamethasone specifically represses iNOS gene expression, among other things, with no acute effect on NOS activity [26]. These pharmacological treatments for IR injury do not enable a conclusion to be drawn regarding the NOS isoform(s) generating the NO that contributes to the injury. However, in view of the extensive evidence for loss of endothelial capacity to produce NO early in reperfusion, iNOS is more likely than eNOS to account for the deleterious level of NO production.

Radiochemical measurement of NOS activities in cell-free muscle homogenates indicated by 8 h of reperfusion Ca2+-dependent NOS activity decreased, whereas Ca2+-independent NOS levels (iNOS) were increased. Dexamethasone inhibited the increase in iNOS activity, but had no detectable effect on Ca2+-independent NOS activity. RT-PCR of muscle extracts provided evidence of iNOS expression at 8 h after the commencement of reperfusion. The appearance of the iNOS message was prevented in rats treated with dexamethasone. These observations indicate the potential of iNOS to contribute to the IR injury. In renal ischaemia, anti-sense oligonucleotides to iNOS ameliorated the reperfusion injury, implicating NO [7]. In contrast, the beneficial effects of NO donors and supplemental l-arginine in myocardial ischaemia are consistent with the notion that NO attenuates IR injury [27–30], and there is a similar interpretation of data from a series of studies of IR injury to mesentery and gut tissue [31–33]. The reason for organ-specific effects of NO in IR injury is not clear, but may relate to the variable importance of neutrophils, since there is evidence that endogenous NO inhibits neutrophil activation and adhesion to endothelium [34–37]. Our studies [38] and those of Skjeldal et al. [39, 40] suggest that neutrophil infiltration is not an important feature of IR-induced necrosis, but there is evidence that neutrophils contribute to the acute (<4 h) oedema response of skeletal muscle [41, 42]. However, neutrophils are accorded a central role in IR injury to myocardial and other tissues [43–45]. It remains to be established which of the deleterious effects of NO, including direct and indirect cytotoxic actions through conversion into the potent oxidant, peroxynitrite [10], account for its contribution to IR-induced necrosis.

The protective effects of dexamethasone do not add or detract from the evidence for a role for NOS in the IR injury. Dexamethasone has a multifaceted anti-inflammatory action, including inhibition of iNOS induction [26]. We confirmed that dexamethasone inhibited iNOS induction in the IR injured skeletal muscle. Thus, the protective effects of dexamethasone and L-NAME could be explained by their inhibition of the contribution of post-ischaemically produced NO to skeletal muscle IR injury. However, the contrast between the effects of L-NAME and dexamethasone at 8 h of reperfusion indicates that dexamethasone, administered at this
late stage in the reperfusion, acts on other components of IR injury in addition to enzymic NO production. Interestingly, a recent report indicates that non-enzymic production of NO through reduction of nitrite may predominate following long periods of ischaemia [46]. It is clear that the anti-inflammatory glucocorticoid has many actions, independent of repression of iNOS, that could explain protection from IR injury. These include: inhibition of activation of the transcription factor NF-kB [47] and consequent decreased expression of leukocyte/endothelial cell adhesion molecules that have been implicated in IR-induced oedema [3], but not necrosis [38]; decreased production of arachidonic acid-derived inflammatory mediators and platelet-activating factor, the latter having been implicated in IR-induced necrosis [48]; and repression of cytokine genes [49] including tumour necrosis factor-α and interleukin-1, which have also been implicated in the IR injury to skeletal muscle [2].

Interestingly, a recent report indicates that there are later opportunities for effective treatment of IR-injured tissue, for example up to 3 h using L-NAME or up to 8 h using dexamethasone after reperfusion. The clinical use of dexamethasone or another glucocorticoid would be a major advance in the treatment regimens available for IR injury to skeletal muscle, since the only drugs occasionally used include anti-platelet agents (e.g. dipyridamole), anti-coagulants (e.g. heparin) and thrombolytic agents (e.g. urokinase), which facilitate reperfusion rather than directly inhibit the reperfusion injury.

Our results indicate the potential for drug treatment of IR injury to skeletal muscle even after several hours of reperfusion. However, administration of either a glucocorticoid or an NOS inhibitor before ischaemia may exacerbate the reperfusion injury.

ACKNOWLEDGMENT

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