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The Effect of Indomethacin on the Production of Eicosanoids and Edema during Ischemia-Reperfusion Injury in Skeletal Muscle

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During reperfusion of skeletal muscle after ischemia, lipid mediators, mainly eicosanoids, are released and may have a role in the pathogenesis of reperfusion injury. To validate the role of eicosanoids in the ischemia-reperfusion induced functional deficits in skeletal muscle, we compared muscle edema and the changes of eicosanoid concentration in the rat hind limb after ischemia-reperfusion injury by application of tourniquet. After 4 hours of ischemia, reperfusion was established for 4 hours by releasing tourniquet. To assess tissue damage, edema, and wet/dry weight ratios were determined and the eicosanoid concnentrations were measured by the HPLC. The muscle edema and the release of cyclooxygenase metabolites were not induced by the ischemia itself rather they were significantly increased by reperfusion. Indomethacin treatment ameliorated limb edema and decreased the release of 6-keto-$PGF_{1\alpha}$, thromboxane B_2, and PGE_2 induced by reperfusion. But the inhibitory effect of indomethacin on edema (35%) was relatively low than the inhibitory effect on release of cyclooxygenase metabolites (up to 69%) by reperfusion. These results support the view that cyclooxygenase products may play a significant role in the formation of muscle injury by ischemia-reperfusion and suggest that nonsteroidal antiinflammatory agents might be partially beneficial to the management of acute limb ischemia-reperfusion injury.

Key Words: Ischemia, Reperfusion injury, Sketal muscle, Indomethacin, Eicosanoid, Prostaglandins

INTRODUCTION

Ischemia and reperfusion of sketal muscle occurs in trauma, thromboembolism, elective vascular surgery and in reconstructive surgery by means of transplantation of muscle containing cutaneous flaps (Kendrick et al, 1987). After long standing ischemia, reperfusion causes injury to muscle and nerve in the ischemic area. The reactive oxygen radicals, and peroxidized lipid mediators released during reperfusion and lipid peroxidation are thought to cause direct damage to cell membrane and induce the release of arachidonic acid and from which vasoactive eicosanoids may be produced, for example thromboxane A_2 and leukotriene B_4 (LTB_4) (Korthuis et al, 1985; Lindsay et al, 1988). These mediators effect critical structural and functional changes in endothelial cells and myocytes, resulting in enhanced capillary permeability and interstitial edema, and cytolysis leading to tissue damage (Meerson et al, 1982; Homer-Vanniasinkam & Gough, 1994). In addition, thromboxane A_2 is a potent vasoconstrictor that may effect changes in microvascular perfusion while LTB_4 facilitates neutrophil recruitment within the perfused muscle. Arachidonic acid, most abundant componenet of unsaturated fatty acids in the cell membrane, is released from the cell membrane during ischemia with increased cyclooxygenase (COX) and lipoxygenase (LO) activity. During reperfusion, prostaglandins (PGs) and thromboxanes (TXs) are released by the action of COX. Leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) are released by LO. These eicosanoids play an important role as

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peroxidized lipid, especially known to act diversely with each metabolite during reperfusion (Lelcuk et al, 1985; Flecher, 1993). These lipid peroxidates have longer duration of action than other free radicals, and easily move from one site to another with tissue destruction (Girotti, 1985). Despite all of these metabolites are released from the reperfused limb, a definite role for these metabolites in the pathogenesis of reperfusion injury and their importance in promoting muscle edema and damage have not been established in whole-limb ischemia.

Therefore, by observing the change of muscle edema during ischemia and reperfusion, and by simultaneously making a quantitative analysis of eicosanoids, we evaluated the release of eicosanoids, especially COX metabolites, by ischemia-reperfusion insult. We also compared the effects of cyclooxygenase inhibitor, indomethacin on edema and eicosanoid levels to see which eicosanoids have roles in edema formation related with ischemia-reperfusion injury of the skeletal muscle.

METHODS

Experimental protocol

Male Sprague-Dawley rats weighing between 300 and 350 gm were anesthetized by intraperitoneal injection of chloral hydrate (380 mg/kg). A touniquet was applied around the proximal thigh and body temperature of the anesthetized animals was maintained at 36.5~37°C using homeothermic pad (Homeothermic blanket control unit; Harvard, MN, USA). Indomethacin mixed in normal saline with a few drops of tween 80, was used as suspension, and was injected subcutaneously 20 mg/kg on the back one hour before ischemia.

Ischemia-reperfusion insult in rat hind limb

A pneumatic tourniquet was placed around the right thigh with the cuff inflated to two times the systolic pressure, 220~240 mmHg, producing ischemia (Whetzel, 1997). The extent of ischemia was observed by skin paleness and the decrease in temperature. Lower leg temperature was measured during ischemia and reperfusion by inserting a contact thermometer probe (HI 93510, Hanna Instruments, Singapore) under the cuff in the distal part. It was 32~33°C before ischemia, but dropped off and was maintained at 26~27°C during ischemia. After four hours of ischemia under anesthesia, reperfusion was initiated by deflating the cuff.

4 groups were studied, and 8 to 10 rats were used in each group.

1. *Group 1*: Received anesthesia and cuff wrapped without induction of right hind limb ischemia (sham).
2. *Group 2*: Subjected to tourniquet ischemia of right hind limb for four hours, without reperfusion (Ischemia only).
3. *Group 3*: Saline injection and subjected to tourniquet ischaemia of right hind limb for four hours, followed by reperfusion for four hours (Ischemia-reperfusion).
4. *Group 4*: Pretreatment with indomethacin, and subjected to tourniquet ischemia of right hind limb for four hours, followed by reperfusion for four hours (Ischemia-reperfusion with indomethacin pretreatment).

At the end of each experiment, the whole tibialis anterior muscle was exposed. In the mid portion, a horizontal incision was made in order to take two biopsies (about 1 gm). One was used for edema measurement, the other was immediately weighed, frozen in liquid nitrogen and stored at −70°C for later analysis of eicosanoids

Edema assessment

The tibialis muscle biopsy obtained above was weighed (wet weight), dried at 80°C for 48 hours in dry oven and the dry weight was measured to calculate edema ratio (wet/dry weight).

HPLC analysis of eicosanoids

Cyclooxygenase metabolites were analyzed according to the method described by Kim's et al (1998). Skeletal muscle biopsies obtained above were mixed with 0.5 ml of cold 100% methanol with 0.1% acetic acid. After homogenization with a Polytron tissue homogenizer (Ultra Turrax, IKA Lab, Germany), the homogenates were centrifugated and the supernatants were brought to a 25% methanol concentration by the addition of 0.1% acetic acid. Two ml of C_{18} Sep-Pak column (Waters Co, IL, USA) was prewashed with 5 ml of 0.1% acetic acid in methanol and 5 ml of 0.1% acetic acid. Then the above sample was loaded and washed with 5 ml of 0.1% acetic acid and 5 ml

of 0.1% acetic acid containing 25% methanol. Cyclooxygenase metabolites were eluted with 0.1% acetic acid containing 90% methanol. The eluents were evaporated by a vacuum concentrator (SpinVac, Hanil Co, Korea) and reconstituted with a 100 μl of 50% methanol. Fifty μl was injected into a HPLC column. To analyze the cyclooxygenase metabolites, Beckman Ultrasphere C_{18} (5 μm, 250×4.6 mm) column was used. The mobile phase was acetonitrile/water (32.8/67.2, v/v), and the pH adjusted to 3.3 with phosphoric acid. A Pharmacia LKB 2141 UV detector was used and peaks were monitored at 195 nm with a 1.5 ml/min flow rate to detect PGs and Txs.

Statistical analysis

The data were presented as means±SEM, and were studied using one-way analysis of variance (ANOVA) in combination with Newman-Keuls multiple range test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Tibialis anterior muscle edema by ischemia and reperfusion

The four hour ischemia only group showed no evidence of muscle swelling (3.11±0.14 vs. 3.05±0.058). In reperfused animals after ischemia, marked hind limb swelling occurred as compared to the control (52%: 4.28±0.17 vs 2.8±0.042). Indomethacin pretreatment reduced muscle swelling significantly up to 35% by ischemia-reperfusion injury (3.86 ±0.124), but wet/dry weight ratio was still high compared to control group (Fig. 1).

Ischemia and reperfusion-induced eicosanoids release in rat hind limb

The concentrations of 6-keto-$PGF_{1\alpha}$, the final product of PGI_2, in skeletal muscle showed no significant difference between the control (474.16±41.88 ng/g) and the ischemia only group (596±28.28 ng/g). In the ischemia-reperfusion group, 6-keto-$PGF_{1\alpha}$ showed 151% increase (1191.17±101.15 ng/g) compared to the control. In the indomethacin pretreatment group this increase was markedly inhibited (718.72±68.19 ng/g) (Fig. 2). The concentrations of thromboxane B_2, the final product of thromboxane A_2, were not significantly different between the control (352.01±72.62 ng/g) and the ischemia only group (395.72±48.56

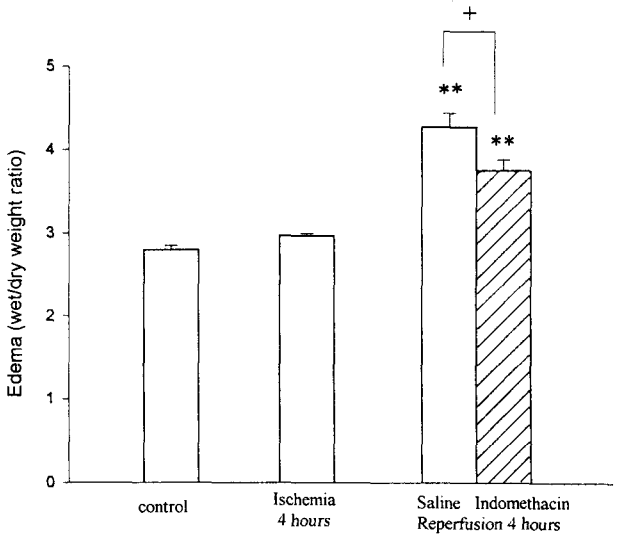

Fig. 1. Wet-to-dry weight of skeletal muscles after 4 hours of ischemia and 4 hours of reperfusion in rat tibialis anterior. Administration of saline and indomethacin (hatched bar) 20.0 mg/kg i.p. 1 hour before ischemia. Values are means SEM. **P<0.01 vs. control group; $^{+}$P<0.05 vs. saline-treated group.

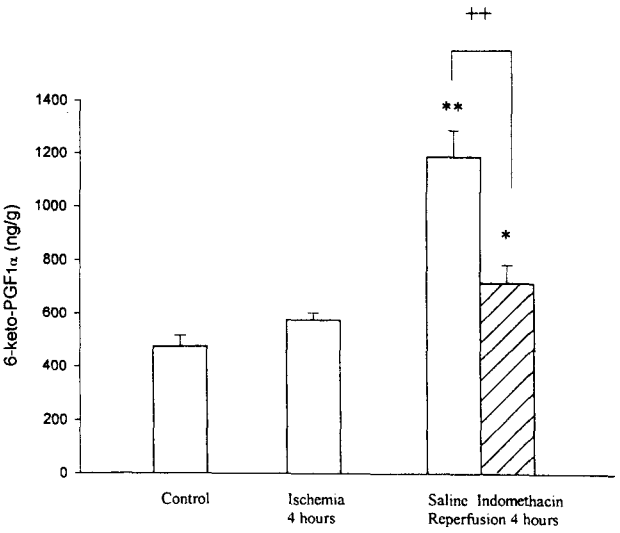

Fig. 2. The concentration of 6-keto-PGF_1, a stable metabolite of prostacyclin (PGI_2), after 4 hours of ischemia and 4 hours of reperfusion in rat tibialis anterior muscle. Administration of saline and indomethacin (hatched bar) 20.0 mg/kg i.p. 1 hour before ischemia. Values are means SEM. *P<0.05 and **P<0.01 vs. control group; $^{++}$P<0.01 vs. saline-treated group.

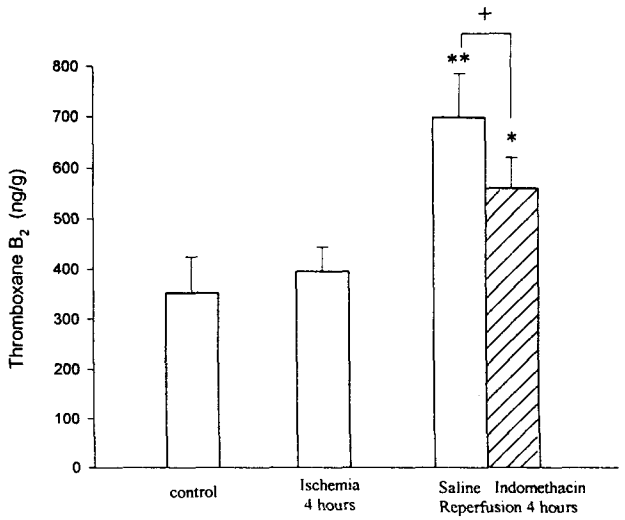

Fig. 3. The concentration of thromboxane B_2, a stable metabolite of of thromboxane A_2, after 4 hours of ischemia and 4 hours of reperfusion in rat tibialis anterior muscle. Administration of saline and indomethacin (hatched bar) 20.0 mg/kg i.p. 1 hour before ischemia. Values are means SEM. *$P<0.05$ and **$P<0.01$ vs. control group; $^{+}P<0.05$ vs. saline-treated group.

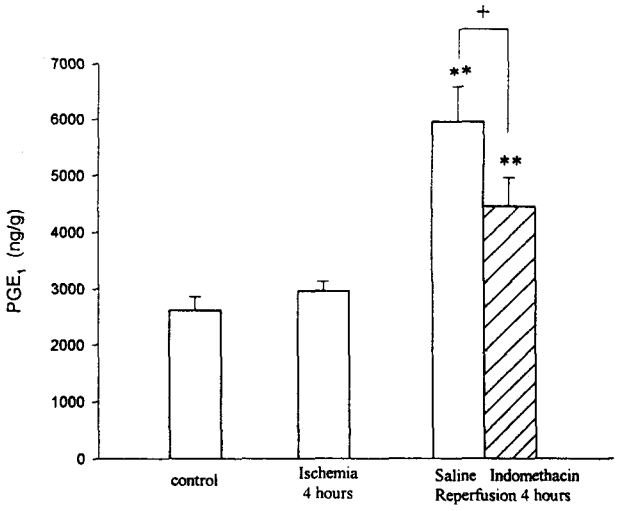

Fig. 4. The concentration of PGE_1 after 4 hours of ischemia and 4 hours of reperfusion in rat tibialis anterior muscle. Administration of saline and indomethacin (hatched bar) 20.0 mg/kg i.p. 1 hour before ischemia. Values are means SEM. **$P<0.01$ vs. control group; $^{+}P<0.05$ vs. saline-treated group.

ng/g). In the ischemia-reperfusion group, it was increased 98% as 698.91±87.28 ng/g, but in the indomethacin pretreatment group, the increase of thromboxane B_2 level (560.54±59.88 ng/g) was significantly inhibited (Fig. 3). PGE_2 concentrations in skeletal muscle were not different between the control (2611±246.77 ng/g) and the ischemia alone (2956.3 ±174.61 ng/g). In the ischemia-reperfusion group, it was increased 128% as 5953.1±632.93 ng/g, but in the indomethacin pretreatment group, the increase of PGE_2 level (4518.72±68.19 ng/g) was significantly inhibited (Fig. 4).

Briefly, in four hours ischemia only group showed+ no significant changes in edema and concentrations of prostaglandins and thromboxane. But edema was prominently increased ($P<0.01$), and the releases of 6-keto-$PGF_{1\alpha}$ ($P<0.01$), PGE_2 ($P<0.01$), and thromboxane B_2 ($P<0.05$) were enhanced in descending order by four hours reperfusion. After indomethacin pretreatment, edema and eicosanoid increased after ischemia-reperfusion of skeletal muscle was inhibited. This action was the same order as above,: 6-keto-PGF_1 ($P<0.01$), PGE_2 ($P<0.05$), and thromboxane B_2 ($P<0.05$), but, edema inhibition was relatively low ($P<0.05$).

DISCUSSIONS

Previous experiments reported that there was little muscular injury and little pathological change during reperfusion after two hours of ischemia, while in six hours of ischemia, irreversible injury was induced so that reperfusion inevitably results in muscular necrosis (Jennische & Hansson, 1986; Skjeldal et al, 1993). Thus, in this experiment, we observed the changes of eicosanoids after four hours of ischemia and four hours of reperfusion. The increase of capillary perfusion after ischemia helps sustain muscular structure, which is generally the aim of muscle flap. However, it is not always beneficial during reperfusion, that is, reperfusion into the ischemic tissue actually causes complex series of reactions that may injure the tissue (Hoch et al, 1991; Seyama, 1993). This paradoxical effect is known to be associated with the formation of reactive oxygen species in the perfused tissue (Mccord et al, 1985; Walker et al, 1987). Proinflammatory mediators enhance circulating granulocyte activity and neutrophil chemotaxis promoting neutrophil-endothelium adhesion to vascular isolation of neutrophils (Muller et al, 1988). From these activated neutrophils, reactive oxygen species and cytotoxic proteolytic enzymes are released and induce extracellular substrate damage and peroxidation of phos-

pholipid layer of cell membrane enhancing microvascular and tissue injury (Kirshinger et al, 1995). Lipid peroxidation has profound effects on cellular structure and function (Crinnion et al, 1993), causing membrane destruction, altered enzyme activity and DNA strand breakage. Activation of phospholipase A_2 induce the release of arachidonic acid and a product of lipid peroxidation, the peroxyl free radical (which causes further membrane peroxidation), and promotes a self-amplifying system of cellular attack culminating in widespread tissue damage (Lindsay et al, 1988). Briefly, as the pathological mechanism, (1) intracellular Ca^{2+} overload, (2) oxygen free radical damage, (3) the change of arachidonic acid metabolism are thought to play important roles (Urbaniak et al, 1997). The above three pathways are extensively interrelated. In particular, arachidonic acid metabolites are so diverse that individual actions are not yet thoroughly revealed, and show tissue specific pathophysiologic actions. Stable metabolites of eicosanoids with biological activities especially thromboxane B_2, metabolite of thromboxane A_2,: 6-keto-$PGF_{1\alpha}$, the marker of PGI_2 production because it is the stable metabolite of PGI_2,: and PGE_2 were quantitated simultaneously. Other prostaglandins, leukotrienes, and HETEs were not detected by HPLC system used. Although the basal and released concentrations of leukotrienes and HETEs in muscle during ischemia-reperfusion were too low to be detected, it should be considered that they can have high activity and cause tissue injury or interact with prostaglandins, thromboxanes or other inflammatory mediators in damaged tissue to aggravate tissue injury.

Water content (wet/dry weight raiio) in ischemia-reperfusion group is 13% more than that of indomethacin treatment group. This difference means that there is a tissue osmotic pressure difference caused by ischemia-reperfusion injury between ischemia-reperfusion group and indomethacin treatment group. The osmotic pressure difference is caused by the muscular protein released from injured muscle fiber or by the increased vascular permeability to plasma protein in ischemia-reperfusion group. Generally the degree of swelling is known to be proportional to tissue damage. Swelling formation is inhibited in indomethacin pretreatment group, so NSAID treatment is thought to have protective effect in such long term ischemic injury as flap manipulation. But, in this experiment the inhibitory effect of swelling by indomethacin is much smaller than the inhibitory effect of eicosanoid formation by the drug. This means that eicosanoids are partially related to tissue ischemia-reperfusion injury and that other active biological systems play bigger roles. Therefore, to establish more effective measures than NSAIDs, further study of the actions of more selective antioxidant, nitric oxide (NO) inhibitor (Liu et al, 1998), lipoxygenase inhibitor, leukotriene receptor antagonist (Lehr et al, 1991), and, thromboxane receptor blocker (Humphrey et al, 1997), and of the measurement of muscle function and viability change in case of concomittent application with NSAID should be investigated.

These results suggest that cyclooxygenase products especially prostacyclin, thromboxane A_2, and PGE_2 are released higher amounts than other eicosanoids by the local tissue injury of ischemia-reperfusion insult, and that nonsteroidal antiinflammatory agents may be partially beneficial to the management of edema formation by acute limb ischemia-reperfusion injury. Further experiments are necessory to evaluate the role of other inflammatory mediators and their interaction with eicosanoids in this injury model.

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