Protective roles of adenosine A₁, A₂A, and A₃ receptors in skeletal muscle ischemia and reperfusion injury

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Submitted 15 July 2007; accepted in final form 1 October 2007


Our objective was to define the function of various adenosine receptor subtypes. N⁶-(R-phenyl-2-propyl)adenosine (R-PIA), an adenosine A₁ receptor agonist of low selectivity, exerted an anti-ischemic effect in a pig latissimus dorsi muscle flap model (28). The adenosine A₁ receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.2 mg/kg) blocked the protection by adenosine in this model. Although these data suggested a role for the adenosine A₁ receptor in mediating protection against ischemia-reperfusion injury in skeletal muscle, it is not known whether other adenosine receptor subtypes also protect skeletal muscle. Activation of the adenosine A₃ receptor has been shown to protect the myocardium against ischemia and reperfusion injury (2, 24, 26). However, the activation of the adenosine A₃ receptor expressed in rodent mast cells stimulates inflammation (30, 37) with a potentially deleterious effect on skeletal muscle. A systematic investigation of the cytoprotective role of adenosine A₁, A₂A, and A₃ receptors in skeletal muscle is needed, along with a genetic approach and a detailed pharmacological characterization of selective agonists and antagonists.

Our objective was to define the function of various adenosine receptor subtypes in skeletal muscle ischemia and reperfusion injury. We used a mouse hindlimb ischemia-reperfusion injury model and demonstrated for the first time a novel anti-ischemic cytoprotective role of the adenosine A₃ receptor. A detailed and specific pharmacological characterization of both A₁ and A₂A receptors was also carried out. We used phospholipase C (PLC)-β2/β3 knockout mice to determine the signaling role of this enzyme in mediating the cytoprotective role of the adenosine receptor subtypes.
MATERIALS AND METHODS

Mouse hindlimb ischemia and reperfusion model. After 2.5- to 3-mo-old wild-type (WT; C57BL6 strain) or PLC-β2/β3 knockout mouse hindlimb ischemia and reperfusion was carried out on 37°C warming pad (Physitemp Instruments, Clifton, NJ) during the reperfusion. After the mice were euthanized by anesthetic overdose, the gastrocnemius muscle was quickly frozen, cut into three slices separated by 2 to 3 mm, and embedded in Shandon Cryomatrix (10% polyvinyl alcohol and 4% polyethylene glycol; Anatomical Pathology, Pittsburgh, PA). Each slice was processed as one 10-μm section on a Thermo Electron/Shandon Cryotome (Anatomical Pathology). fixed in ice-cold acetone, air dried, and washed in phosphate-buffered saline (PBS). Each 10-μm section had seven fields. Gastrocnemius was used because of its high proportion of fast-twitch muscle, which is prone to ischemia and reperfusion injury (1, 11).

Quantification of skeletal muscle injury. Evans blue dye (EBD), prepared as a 1% wt/vol solution to yielding 1 mg of EBD/10 g body wt, was given via a separate intraperitoneal injection 2.5 h before the induction of ischemia. EBD stained only injured muscle, and EBD-positive cells were quantified according to a previously described method (14). The percentage of EBD-positive cells in each field was averaged with those from all seven fields within one 10-μm section. The averaged fraction of EBD-positive cells in each 10-μm section was similar among the three sections. Each 10-μm section was also stained with rabbit polyclonal anti-skeletal muscle actin antibodies (ab15265; Abcam, Cambridge, MA) and goat polyclonal anti-rabbit IgG conjugated with fluorescein isothiocyanate. Sections were mounted, and their cross sections were viewed with fluorescent microscopy (EBD-positive cells via a DM510 filter of 450–490 nm with emission at 520 nm). Each 10-μm section had seven fields. Gastrocnemius was used because of its high proportion of fast-twitch muscle, which is prone to ischemia and reperfusion injury (1, 11). A significant reduction in the extent of injury (data not shown).

RESULTS

Role of adenosine A1 receptors in anti-ischemic skeletal muscle protection. Ischemia followed by reperfusion resulted in significant limb skeletal muscle injury in PBS vehicle-treated mice. The extent of injury was quantified by an increase in the EBD staining of the skeletal myocytes (Fig. 1, A and B). The fraction of total skeletal muscle cross sections that stained positive for EBD was 28 ± 6% (n = 7 mice, means ± SE, Fig. 1B). Administration of the relatively nonselective adenosine receptor agonist R-PIA before ischemia and reperfusion caused a significant reduction in the extent of injury (data not shown). To elucidate the cytoprotective role of different adenosine receptor subtypes, we found that a highly A1 receptor-selective agonist, CCPA, induced a large decrease in the extent of muscle injury (Fig. 1C; EBD-positive area in CCPA-treated mice: 10.8 ± 2%, n = 12 mice vs. PBS vehicle-treated mice: 28 ± 6%, n = 7 mice, P < 0.05). The A1 receptor-selective antagonist DPCPX completely abrogated the cytoprotective response to CCPA (Fig. 1C; DPCPX and CCPA treatment: 22 ± 3.3%, n = 9 mice, P < 0.05 vs. CCPA treatment). The adenosine A3 receptor-selective antagonist MRS-1191 did not affect CCPA-mediated protection (Fig. 1C; MRS-1191 and CCPA treatment: 12.2 ± 2.4%, n = 23 mice, P > 0.05 vs. CCPA treatment), indicating that the adenosine A1 receptor protects skeletal muscle from ischemia-reperfusion injury. The contralateral limb not subjected to ischemia-reperfusion showed no EBD staining (data not shown), indicating an absence of muscle injury in the nonischemic limb.

A novel anti-ischemic protective role of adenosine A3 receptors in skeletal muscle. Our data demonstrated that the A1 receptor agonist CI-IBMECA induced a significant reduction in EBD-positive cells (Fig. 2A; CI-IBMECA treatment: 5.4 ± 2.6%, n = 8 mice, P < 0.05 vs. PBS treatment). This reduction...
was sensitive to antagonism by MRS-1191 (Fig. 2A; MRS-1191 and Cl-IBMECA treatment: 21.5 ± 3.5%, n = 22 mice, P < 0.05 vs. Cl-IBMECA treatment) but not by DPCPX (Fig. 2A; DPCPX and Cl-IBMECA treatment: 4 ± 1.6%, n = 9 mice, P > 0.05 vs. Cl-IBMECA treatment). Neither MRS-1191 (23 ± 4.4%, n = 15 mice) nor DPCPX (19.3 ± 4.4%, n = 8 mice) alone had any effect on the extent of ischemia-reperfusion-induced skeletal muscle injury.

Mice pretreated with the adenosine A2A receptor-selective agonist CGS-21680 showed reduced muscle injury compared with PBS vehicle-treated animals (6.6 ± 3.5%, n = 9 mice, P < 0.05 vs. PBS treatment; Fig. 2A). The protective effect of CGS-21680 was attenuated by DPCPX (DPCPX and CGS-21680 treatment: 14.7 ± 2.3%, n = 10 mice, P > 0.05 vs. DPCPX alone). The A1 antagonist MRS-1191 could not inhibit the CGS-21680-induced skeletal muscle protection (MRS-1191 and CGS-21680 treatment: 2.4 ± 1.25%, n = 8 mice, P < 0.05 vs. MRS-1191 alone). The adenosine A2A receptor-selective antagonist SCH-442416 completely abrogated the CGS-21680-induced protection (Fig. 2A). Animals treated with SCH-442416 and CGS-21680 showed significantly larger EBD-positive areas (26 ± 4%, n = 5 mice, means ± SE) than mice treated with CGS-21680 alone (P < 0.05).

We measured CK levels as another method to quantify skeletal muscle injury induced by ischemia and reperfusion. Cl-IBMECA, CGS-21680, and CCPA were able to reduce these levels when each agonist was administered individually before ischemia and reperfusion (Fig. 2B). CK in Cl-IBMECA-treated mice was 1,840 ± 910 U/L, n = 13 mice. In CCPA-treated mice, CK was 2,340 ± 710 U/L, n = 11 mice. CK in CGS-21680-treated mice was 838 ± 243 U/L, n = 10 mice (P < 0.05 for any agonist vs. vehicle-treated mice, which had a serum CK level of 12,600 ± 3,300 U/L, n = 14 mice). The protection against CK release induced by A1, A2A, or A3 receptors was blocked by an antagonist of each adenosine receptor subtype. In mice treated with MRS-1191 and Cl-IBMECA, serum CK was 14,400 ± 2,900 U/L (n = 15 mice, P < 0.05 vs. Cl-IBMECA alone). Serum CK in mice treated with DPCPX plus CCPA was 11,300 ± 2,200 U/L (n = 9 mice, P < 0.05 vs. CCPA alone). CK in mice treated with SCH-442416 plus CGS-21680 was 15,180 ± 4,420 U/L, n = 8 mice. The data obtained on serum CK activity, derived from the same method of agonist and antagonist administration in the same ischemia-reperfusion injury model, complement those obtained through EBD staining.
Serum CK, as did the A1 receptor agonist CCPA or the A2A receptor agonist MRS-1191 plus Cl-IBMECA, induced skeletal muscle protection. Adult WT mice were injected with various adenosine ligands, subjected to I/R injury, and their skeletal muscle injuries were quantified as described in Fig. 1. A3 receptor agonist Cl-IBMECA caused a reduction in the fraction of EBD-positive areas (PBS treatment: 28 ± 6% EBD-positive area, n = 7; PLC-β2/β3 null mice had 23.4 ± 4% EBD-positive area, n = 9, P > 0.05; Figs. 1C and 3C, respectively).

The A2A agonist CGS-21680 was also able to cause cytoprotection in PLC-β2/β3 null mice (CGS-21680 treatment: 4 ± 1.1%, n = 6 PLC-β2/β3 null mice, P < 0.05 vs. PBS treatment: 23.4 ± 4%, n = 9 PLC-β2/β3 null mice; Fig. 3C). Because the A2A receptor is coupled to stimulation of adenyl cyclase activity and cAMP accumulation, it was not unexpected that the absence of PLC-β2/β3 had no effect on the cytoprotective effect of the A2A receptor. The cytoprotective action of adenosine A2A receptors in skeletal muscle is independent of and separate from the salutary effect mediated by adenosine A1 or A3 receptors in that tissue.

**DISCUSSION**

Ischemia and reperfusion of the skeletal muscle can cause significant injury with deleterious consequences. Effective therapies that reduce such injury will have significant benefits in treatment of trauma, autogenous skeletal muscle transplantation, and vascular and musculoskeletal reconstructive surgery. As with anti-ischemic myocardial protection, adenosine and its receptors have been implicated in protecting the skeletal muscle against ischemia and reperfusion injury. The present study demonstrated for the first time that the adenosine A3 receptor can induce potent cytoprotection of the skeletal muscle against ischemia and reperfusion injury. The adenosine A3 receptor, but not the A1 or A2A receptor, signals via PLC-β2/β3 to achieve its skeletal muscle protective effect.

Several lines of evidence clearly delineate the cytoprotective role of adenosine A1, A2A, and A3 receptors. The highly A1 receptor-selective agonist CCPA decreased skeletal muscle ischemia and reperfusion injury. The protective effect was blocked only by the A1 receptor-selective antagonist DPCPX but not by the A3 receptor-selective antagonist MRS-1191. Conversely, the A3 receptor-selective agonist CI-IBMECA reduced skeletal muscle injury, and this protective effect, although insensitive to blockade by the A1 receptor antagonist DPCPX, was completely abrogated by the A3 receptor antagonist MRS-1191, which was shown to antagonize the adenosine A3 receptor in mice (3, 38, 39). Similarly, the cytoprotective effect of A2A receptor agonist CGS-21680 was selectively blocked by its antagonist SCH-442416 and was insensitive to the A3 antagonist MRS-1191. The A1 antagonist DPCPX, at the current dosage, was able to attenuate the CGS-21680-induced skeletal muscle protection. Several explanations are possible. Given the interaction between A1 and A2A receptors, it is possible that A2A receptor-mediated effect could be potentiated by A1 receptor activation. A positive interaction...
between adenosine A₁ and A₂A receptors was recently demonstrated in rat heart (23). Since DPCPX could inhibit the protective effect of CGS-21680 in the current skeletal muscle ischemia-reperfusion injury model, it is possible that A₁ receptor activation contributed to the CGS-21680-induced skeletal muscle protection. Recent evidence suggests that protein kinase C (PKC) activation can potentiate the adenosine A₂B receptor signaling during reperfusion in the heart (22). Thus another possible explanation is that PKC activation, induced by adenosine A₁ receptor, may also increase the responsiveness of the adenosine A₂A receptor signaling during reperfusion in skeletal muscle. Overall, the present data provided detailed characterization of antagonists and agonists associated with A₁, A₂A, and A₃ receptors in the current in vivo model of skeletal muscle ischemia-reperfusion injury. The study confirmed their selectivity at each adenosine receptor subtype and indicates that the cytoprotection afforded by each receptor agonist was due to activation of that specific receptor.

PLC-β2/β3 deficiency selectively abrogated the protective effect of A₃ receptor agonist Cl-IBMECA and had no effect on CCPA- or CGS-21680-induced protection. It was unlikely that PLC-β2/β3 deficiency affected the bioavailability or pharmacokinetic properties of Cl-IBMECA for the following reason. CCPA and CGS-21680 have similar size and molecular weights as Cl-IBMECA. Both CCPA and CGS-21680 were fully capable of protecting against skeletal muscle injury in the PLC-β2/β3 knockout mice. In WT mice, CGS-21680 induced anti-ischemic skeletal muscle protection in a manner that was insensitive to blockade by MRS-1191 but was completely abolished by the A₂A receptor-selective antagonist SCH-442416. The protective effect of CGS-21680 remained unaffected and intact in PLC-β2/β3 null mice. The exact bioavailability or pharmacokinetic property of Cl-IBMECA in WT or PLC-β knockout mice remains to be determined.

Although PLC-β2/β3 is not involved in mediating the protective effect of A₁ or A₂A receptors in the skeletal muscle, ATP-sensitive K⁺ channels appear to be an important effector mechanism for the anti-ischemic effect of the A₁ receptor (28). The adenosine A₂A receptor serves an important nonredundant role in suppressing immune and lymphoid cells and thus in protecting against inflammatory tissue damage (25, 32, 33). Since activation of adenosine A₂A receptors on CD4⁺ T cells mediated potent protection against renal ischemia-reperfusion injury (12), it is possible that the same mechanism is also responsible for its protection in the skeletal muscle in vivo. Activation of the A₃ receptor in rodent immune cells such as mast cells is proinflammatory and may be damaging (10, 20). A genetic absence or antagonism of adenosine A₃ receptors augmented an increase in coronary flow or hypotension mediated by adenosine or an A₂A receptor agonist (36, 43), pointing to a vasoconstrictive role of the vascular A₃ receptor. Activated mast cells and neutrophils mediate skeletal muscle ischemia-

Fig. 3. The adenosine A₃ receptor signals through PLC-β2/β3 to cause its anti-ischemic skeletal muscle protective response. Adult PLC-β2/β3 null mice were injected with vehicle (n = 9 mice), Cl-IBMECA (n = 8 mice), CGS-21680 (n = 6 mice), or CCPA (n = 10 mice); they were subjected to I/R, and the extent of skeletal muscle injury was subsequently quantified as in WT mice. In PLC-β2/β3 null mice not subjected to I/R, skeletal muscles did not show any EBD staining. A: a typical EBD staining in a vehicle-injected PLC-β2/β3 null mouse following I/R is shown. The extent of EBD staining was similar to that obtained in vehicle-injected WT mice after I/R (see Fig. 1C, P > 0.05). B: a representative EBD staining in a Cl-IBMECA-treated mouse is shown. C: treatment with CCPA or CGS-21680 reduced EBD staining, but Cl-IBMECA did not. Average EBD staining (means ± SE) of skeletal muscle sections from PLC-β2/β3 null mice following treatment with vehicle or adenosine ligand is shown. *P < 0.05, CCPA, CGS-21680 vs. either PBS or Cl-IBMECA. P > 0.05, PBS vs. Cl-IBMECA; P > 0.05, CCPA vs. CGS-21680.
reperfusion injury (6, 16, 17). The present data could not determine whether the adenosine A3 receptor-mediated protection was due to direct activation of A3 receptors on the skeletal muscle or the result of an anti-inflammatory action of A3 receptors on the immune cells. EBD is a dye that accumulates in injured tissues as a result of an increase in vascular permeability (35) and a disruption of sarcosomal integrity of the tissue (such as muscle) supplied by the vasculature. It is possible that a decrease in EBD staining was due to a decrease in vascular permeability induced by one or all of the adenosine receptor subtypes studied. Differentiating a protective effect of adenosine receptor subtypes at the levels of vasculature, circulating immune cells, and skeletal muscle is needed. Bone marrow transplant from adenosine receptor knockout mice, or possible creation of vascular or skeletal muscle specific knockout of adenosine receptor subtypes, would provide a more definitive answer to this question.

That the adenosine A3 receptor exerts a potent cytoprotective effect in mouse skeletal muscle is consistent with its cardioprotective action in the mouse heart (19). Although PLC is currently shown to have an important role in mediating the cardioprotective action in the mouse heart (19), although several plausible explanations are offered. First, (29). The reasons for this apparent difference are not clear; however, several plausible explanations are offered. First, species and age differences (chick embryos vs. adult mouse) may be important. Second, the coupling of adenosine A3 receptors to PLC vs. PLD may be different in skeletal than in cardiac muscles. Third, our skeletal muscle ischemia-reperfusion injury preparation was an in vivo and intact animal model, whereas the model used by Parsons et al. (29) was an isolated cell culture model. The present gene knockout approach rendered all cells completely deficient in PLC-β2/β3, including skeletal muscle and circulating immune cells capable of mediating anti-and proinflammatory. It is possible that PLC mediated an anti-inflammatory effect of A3 receptors on circulating immune cells. In this scenario, knockout of PLC would eliminate the anti-inflammatory effect of A3 receptors on immune cells and thus abrogated their cytoprotective effect on skeletal muscles.

The combined use of receptor pharmacological tools and a gene ablation approach delineated, for the first time, a distinct anti-ischemic protective role of adenosine A1, A2A, and A3 receptor subtypes in skeletal muscle. Although both adenosine A1 and A2A receptors have shown anti-ischemic protective properties, agonists at either receptor caused pronounced decreases in blood pressure or heart rate. Our data provide convincing evidence that the adenosine A1 receptor is a novel cytoprotective receptor in skeletal muscle. Because the A3 receptor agonist is not associated with cardiac or hemodynamic depression (2), the A3 receptor represents a potential therapeutic target because of its ability to ameliorate skeletal muscle injury.

ACKNOWLEDGMENTS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. We thank Dr. Kevin Campbell for useful comments and advice.

REFERENCES


ADENOSINE RECEPTOR SUBTYPES IN SKELETAL MUSCLE PROTECTION