Attenuation of Ecto-5′-nucleotidase Activity and Adenosine Release in Activated Human Polymorphonuclear Leukocytes

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To examine whether activation of polymorphonuclear leukocytes attenuates release of adenosine through attenuation of their own ecto-5′-nucleotidase activity, human polymorphonuclear leukocytes were incubated with and without exposure to either N-formyl-methionyl-leucyl-phenylalanine (FMLP) or complement C5a. Ecto-5′-nucleotidase activity of polymorphonuclear leukocytes was attenuated by both FMLP and complement C5a (22.7±3.6 vs 9.7±2.6 nmol/min per 10⁶ cells at 10⁻⁸ M FMLP, P<.05; 21.5±2.2 vs 10.2±1.2 nmol/min per 10⁶ cells at 5×10⁻⁷ g/mL complement C5a, P<.001), whereas cytosolic 5′-nucleotidase activity was not affected by either FMLP or complement C5a. These reductions of ecto-5′-nucleotidase activity that were caused by both FMLP and complement C5a were dose and time dependent and were inhibited by superoxide dismutase. Desferrioxamine did not inhibit the decreases in ecto-5′-nucleotidase. In accordance with the decreases in ecto-5′-nucleotidase activity, release of adenosine was attenuated in the FMLP-pretreated and complement C5a-pretreated polymorphonuclear leukocytes, which were restored by concomitant administration of superoxide dismutase. The viability of FMLP-pretreated and complement C5a-pretreated polymorphonuclear leukocytes was markedly decreased compared with the untreated group after 60 minutes of hypoxia followed by 60 minutes of reoxygenation. Thus, we conclude that (1) activation of polymorphonuclear leukocytes attenuates their own ecto-5′-nucleotidase activity and thereby reduces adenosine release, (2) reduction of ecto-5′-nucleotidase activity is attributable to generated superoxide anion in polymorphonuclear leukocytes, and (3) viability of polymorphonuclear leukocytes after hypoxia and reoxygenation largely depends on the extents of decreases in ecto-5′-nucleotidase activity. (Circulation Research 1993;73:524-533)

KEY WORDS • adenosine • superoxide dismutase • superoxide anion • N-formyl-methionyl-leucyl-phenylalanine • complement C5a • hypoxia

Adenosine has been reported to modify several key cellular activities in the heart.1-3 Adenosine relaxes vascular smooth muscles,4,5 inhibits platelet aggregation6,7 and generation of superoxide anion from polymorphonuclear leukocytes,8-10 and attenuates increases in myocardial contractility due to noradrenaline,11,12 all of which may synergistically attenuate ischemia and reperfusion injury.13,14 Most of all, the deleterious effects of activated polymorphonuclear leukocytes have been recently focused as the potential cause of ischemic and reperfusion injuries.15-20 Adenosine is released not only from cardiomycocytes but also from polymorphonuclear leukocytes,21,22 which may attenuate the activation of polymorphonuclear leukocytes. However, there is no clear consensus whether activation of polymorphonuclear leukocytes attenuates or augments release of adenosine from polymorphonuclear leukocytes. Because our24 and other25 studies have revealed that superoxide anion and lipid peroxidation deactivate ecto-5′-nucleotidase and attenuate adenosine release, activated polymorphonuclear leukocytes, in which superoxide anion is massively generated, may reduce their own 5′-nucleotidase activities. If this is the case, activation of polymorphonuclear leukocytes leads to further attenuation of adenosine release through decreased 5′-nucleotidase activity.26

To test this idea, we measured 5′-nucleotidase activities and adenosine release with and without activation of polymorphonuclear leukocytes. Furthermore, we tested whether the reduction of ecto-5′-nucleotidase activity is attributable to superoxide anion generated from polymorphonuclear leukocytes. Finally, we tested the viability of polymorphonuclear leukocytes with reduced 5′-nucleotidase activity after hypoxia and reoxygenation.

Materials and Methods

Materials
N-Formyl-methionyl-leucyl-phenylalanine (FMLP), complement C5a, desferrioxamine, and cacodylic acid were obtained from Sigma Chemical Co, St Louis, Mo. Human recombinant superoxide dismutase (SOD, 5340 IU/mg, ≥99% purity) and 2′-deoxycoformycin were provided by Ube Industries, Tokyo, Japan, and Yamasa

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Two milliliters ofuffy coat was layered over 3 mL of 5% isotonic colloidal polyvinylpyrrolidone–coated silica (Percoll, Pharmacia, Uppsala, Sweden) solution and centrifuged at 1600g for 20 minutes. The polymorphonuclear leukocyte–rich bands on the erythrocytes were lysed with 0.15 M NH₄Cl. Polymorphonuclear leukocytes were resuspended in saline in the range of 1.5 to 2.5×10⁷ cells/mL. The solution contained (mM) NaCl, 130; KCl, 5; MgCl₂, 1.2; HEPES, 10; CaCl₂, 1.3; sodium acetate, 20; and glucose, 10; with 100% O₂ bubbling. The pH of the solution was normally adjusted with NaOH to 7.40 at 37°C. The suspension prepared in this method contained 96±3% polymorphonuclear leukocytes. The contaminations of mononuclear and platelets were less than 2%, respectively. The vitality test for polymorphonuclear leukocytes was performed by Trypan blue dye exclusion. Viability in separated polymorphonuclear leukocytes was more than 95%.

**Experimental Protocols**

Protocol 1: Effects of FMLP on ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities of polymorphonuclear leukocytes. After polymorphonuclear leukocyte isolation, we added 10⁻⁶ M FMLP to the solution containing 1.5 to 2.5×10⁷ polymorphonuclear leukocytes. First, we observed the time courses of changes in the ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities after an exposure to 10⁻⁶ M FMLP. Second, we changed the dose of FMLP from 10⁻⁶ to 10⁻⁵ M. To obtain the dose–response relation between FMLP and 5'-nucleotidase activities, we observed ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities at 15 minutes after an exposure to each dose of FMLP because the present study revealed that a 15-minute period is necessary to reach the stable condition for changes in ecto-5'-nucleotidase activity (Fig 1).

Protocol 2: Effects of complement C5a on ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities of polymorphonuclear leukocytes. After the isolation of polymorphonuclear leukocytes, we added complement C5a (5.0×10⁻³ g/mL) to the solution containing 1.5 to 2.5×10⁷ polymorphonuclear leukocytes. First, we observed the time courses of changes in the ecto-5'-
nucleotidase and cytosolic 5'-nucleotidase activities after an exposure to complement C5a (5.0x10^{-7} g/mL). Second, we changed the dose of complement C5a from 5.0x10^{-8} to 5.0x10^{-6} g/mL. To obtain the dose-response relation between complement C5a and 5'-nucleotidase activities, we observed ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities at 30 minutes after an exposure to each dose of complement C5a because the present study revealed that a 30-minute period is necessary to reach the stable condition (Fig 3).

Protocol 3: Effects of SOD and desferrioxamine on decreases in ecto-5'-nucleotidase activity due to an exposure to either FMLP or complement C5a. We tested whether SOD blunts the decreases in 5'-nucleotidase activity of activated polymorphonuclear leukocytes due to either FMLP or complement C5a. We performed comparable experiments as in protocol 2 under concomitant exposure to SOD (0.1 mg/mL), ie, (1) time courses of changes in ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities after an exposure to either 10^{-6} M FMLP or complement C5a (5.0x10^{-7} g/mL) under administration of SOD and (2) the dose-response relations between 10^{-6} to 10^{-3} M FMLP and 5'-nucleotidase activities and between complement C5a (5.0x10^{-5} to 5.0x10^{-6} g/mL) and 5'-nucleotidase activities under the administration of SOD.

When polymorphonuclear leukocytes are activated, hydrogen peroxide may be generated via the Haber-Welsh reaction and may additionally affect 5'-nucleotidase activities. To test this possibility, we examined whether an exposure to desferrioxamine (1.5x10^{-4} M), which inhibits hydrogen peroxide generation, blunts the decreases in ecto-5'-nucleotidase activity due to exposures to 10^{-6} to 10^{-3} M FMLP.

Protocol 4: Adenosine release of polymorphonuclear leukocytes with and without an exposure to FMLP. To ascertain that the decreases in ecto-5'-nucleotidase activity attenuate adenosine release, we measured adenosine concentration of solution containing 1.5 to 2.5x10^{7} polymorphonuclear leukocytes pretreated with and without an exposure to 10^{-6} M FMLP for 30 minutes. Furthermore, adenosine concentration was measured in the solution containing polymorphonuclear leukocytes pretreated with and without 10^{-5} M FMLP under SOD treatment (0.1 mg/mL). After the pretreatment with FMLP in the presence or absence of SOD, the polymorphonuclear leukocytes were washed gently twice in the normal solution, and we obtained the time courses of changes in adenosine concentration after addition of 10 μM 2'-deoxycoformycin for inhibition of adenosine deaminase, 1 μM 5'-iodotubercidin for inhibition of adenosine kinase, and 0.02% dipyridamole for
inhibition of adenosine uptake into the cellular components. We measured adenosine release from polymorphonuclear leukocytes in normoxic (O_2, 521±17 mm Hg) and hypoxic (O_2, 26±3 mm Hg) conditions, which were obtained by bubbling the solution with 100% O_2 and 100% N_2, respectively.

Protocol 5: Changes in viability and shapes of polymorphonuclear leukocytes pretreated with FMLP and complement C5a and in the presence or absence of SOD after hypoxia and reoxygenation. Although activation of polymorphonuclear leukocytes decreases their own ecto-5'-nucleotidase activity and thereby attenuates adenosine release during hypoxic conditions, this attenuation of adenosine release does not necessarily cause cellular injury during hypoxia and reoxygenation. Polymorphonuclear leukocytes were treated either with FMLP (10^{-6} M) or complement C5a (5.0x10^{-8} g/mL) in the presence or absence of SOD (0.1 mg/mL) for 60 minutes and washed gently twice in the normal solution. These pretreated cardiomyocytes were then incubated in the hypoxic solution (O_2, 31±4 mm Hg) for 60 minutes followed by 60 minutes of reoxygenation (O_2, 554±21 mm Hg). The viability test for polymorphonuclear leukocytes was performed by Trypan blue dye exclusion during control conditions, at 60 minutes of hypoxia, and at 60 minutes of reoxygenation.

For assessment of changes in the shape of polymorphonuclear leukocytes, the cell suspension was obtained during control conditions, during exposure to and washout of chemicals, at 60 minutes of hypoxia, and at 60 minutes of reoxygenation. The cell suspension was added dropwise to 10 mL cold (4°C) 1% glutaraldehyde in 0.1 M cacodylic acid. The glutaraldehyde solution was mixed constantly while the cells were being added. After remaining in the cold glutaraldehyde solution for 1 hour, the cells were washed and resuspended in 0.1 mL distilled water. The polymorphonuclear leukocytes

FIG 4. Graphs showing the relation between doses of complement C5a (5.0x10^{-8} to 5.0x10^{-6} g/mL) and ecto-5'-nucleotidase (A) and cytosolic 5'-nucleotidase (B) activities in polymorphonuclear leukocytes. Ecto-5'-nucleotidase activity was significantly (P<.001) reduced when the dose of complement C5a was increased. In contrast, cytosolic 5'-nucleotidase activity was not affected by complement C5a.

FIG 5. Time courses of changes in ecto-5'-nucleotidase (A) and cytosolic 5'-nucleotidase (B) activities after an exposure to 10^{-8} M N-formyl-methionyl-leucyl-phenylalanine (FMLP) with and without superoxide dismutase (SOD) treatment. SOD treatment significantly (P<.05) restored the ecto-5'-nucleotidase activity, whereas SOD did not change the cytosolic 5'-nucleotidase activity compared with the data in Fig 1. SOD itself did not affect ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activity, as is evident from the data at time 0.
were classified according to their shape. Cells were categorized spherical or nonspherical. The spherical category included round cells and cells shaped generally round with some ruffled membrane. The nonspherical category included cells whose overall form was oval to elongated and bipolar.29

**Adenosine Measurement**

The method of adenosine measurements has been reported previously.5,29,30 To obtain the time courses of adenosine release in polymorphonuclear leukocytes, we added EDTA (500 mM) and trichloroacetic acid (10%) to block further production and degradation of adenosine. After centrifugation, the supernatant was obtained, and the adenosine content was determined by radioimmunoassay. Adenosine in the solution (100 μL) was succinylated by 100 μL dioxane containing succinic acid anhydride and trimethylamine. After a 20-minute incubation, the mixture was diluted with 100 μL adenosine 2',3'-O-disuccinyl-3-[125I]iodotyrosine methyl ester (0.5 pmol) and 100 μL diluted antiadenosine serum. The mixture was kept in a cold water (4°C) bath for 18 hours, and the second antibody solution (500 μL goat anti-rabbit immunoglobulin G antiserum) was added. After incubation at 4°C for 1 hour, untreated materials were removed by centrifugation at 3000 rpm at 4°C for 20 minutes. The radioactivity remaining in the tube was counted by a gamma counter. The amount of adenosine degradation during this blood-sampling procedure has been reported to be negligible.5,29,30

**Measurement of 5'-Nucleotidase Activity**

After the experiments, the polymorphonuclear leukocytes were homogenized for 5 minutes in 10 vol ice-cold 10 mM HEPES-KOH buffer (pH 7.4) containing 0.25 M sucrose, 1 mM MgCl2, and 1 mM mercaptoethanol. The crude homogenate was strained through a double-layered nylon sieve and again homogenized for 1 minute. For preparation of a crude membrane fraction, part of the homogenate was centrifuged at 1000g for 10 minutes. The resultant pellet was washed three times and finally resuspended in the HEPES-KOH buffer. For preparation of the cytosolic fraction, the remaining part of the homogenate was first centrifuged at 3000g for 10

**Fig 6.** Graphs showing the relation between doses of N-formyl-methionyl-leucyl-phenylalanine (FMLP) and ecto-5'-nucleotidase (A) and cytosolic 5'-nucleotidase (B) activities with and without superoxide dismutase (SOD) treatment in polymorphonuclear leukocytes. The reduction of ecto-5'-nucleotidase activity due to FMLP administration was restored (P<.05) by SOD treatment. In contrast, cytosolic 5'-nucleotidase activity was not affected by FMLP and SOD. The data of both ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities in FMLP-treated polymorphonuclear leukocytes without SOD treatment are the same as in Fig 2.

**Fig 7.** Graph showing the relation between doses of complement C5a and ecto-5'-nucleotidase (A) and cytosolic 5'-nucleotidase (B) activities with and without superoxide dismutase (SOD) treatment in polymorphonuclear leukocytes. The reduction of ecto-5'-nucleotidase activity due to C5a administration was restored (P<.05) by SOD treatment. In contrast, cytosolic 5'-nucleotidase activity was not affected by complement C5a and SOD.
minutes, and the supernatant was centrifuged again at 200,000g for 1 hour. The membrane and cytosolic fractions were dialyzed at 4°C for 4 hours against 10 mM HEPES-KOH (pH 7.4) containing 1 mM MgCl₂, 1 mM mercaptoethanol, and 0.01% activated charcoal and then divided into aliquots that were immediately frozen and stored at -80°C.

The activity of 5'-nucleotidase was assessed by the enzymatic assay technique and was described in moles per minute per 10⁷ cells. 5'-Nucleotidase activities of the membrane and cytosolic fractions were defined as ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities, respectively.

**Statistical Analysis**

Statistical analysis was performed with paired and unpaired t tests. Two-way analysis of variance was also performed to test the differences in time courses and dose-response curves. A post hoc Scheffe's test was used to determine significance for group pairs that exhibited statistically significant differences. All values were expressed as mean±SEM, and P<.05 was considered significant.

**Results**

An exposure to 10⁻⁶ M FMLP decreased ecto-5'-nucleotidase activity of polymorphonuclear leukocytes from 22.7±3.6 to 9.7±2.6 nmol/min per 10⁷ cells at 15 minutes (Fig 1, A). This decrease in ecto-5'-nucleotidase activity continued for 15 minutes after an exposure to 10⁻⁶ M FMLP and thereafter became stable. In contrast, cytosolic 5'-nucleotidase activity was not changed by an exposure to 10⁻⁶ M FMLP (Fig 1, B). Fig 2 shows the dose-response relation between 10⁻⁶ to 10⁻³ M FMLP and 5'-nucleotidase activities. Ecto-5'-nucleotidase activity of polymorphonuclear leukocytes was decreased by FMLP. However, cytosolic 5'-nucleotidase activity was minimally altered by FMLP. An exposure to complement C5a (5.0x10⁻⁸ g/mL) also decreased ecto-5'-nucleotidase activity, whereas it did not decrease cytosolic 5'-nucleotidase activity (Fig 3). Fig 4 shows the dose-response relation between complement C5a (5.0x10⁻⁹ to 5.0x10⁻⁶ g/mL) and 5'-nucleotidase activities. Ecto-5'-nucleotidase activity of polymorphonuclear leukocytes was dose-dependently decreased by complement C5a. However, cytosolic 5'-nucleotidase activity was not altered by administration of complement C5a.

To test the idea that superoxide anions generated from activated polymorphonuclear leukocytes are responsible for the attenuation of their ecto-5'-nucleotidase activity due to FMLP and complement C5a, ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities were observed in the presence of SOD. Interestingly, as shown in Fig 5, SOD blunted the decreases in ecto-5'-nucleotidase activity caused by FMLP. Fig 6 shows the dose-response relations between 10⁻⁸ to 10⁻⁵ M FMLP and 5'-nucleotidase activity under SOD treatment. The decreases in ecto-5'-nucleotidase activity caused by FMLP were completely abolished by SOD. Fig 7 shows the dose-response relations between complement C5a (5.0x10⁻⁹ to 5.0x10⁻⁶ g/mL) and 5'-nucleotidase activities under SOD treatment. The decreases in ecto-5'-nucleotidase activity caused by complement C5a were also abolished by SOD treatment. However, the decreases in ecto-5'-nucleotidase activities caused by FMLP were not altered by desferrioxamine treatment (Fig 8). These results indicate that the reduction of ecto-5'-nucleotidase activity in activated polymorphonuclear leukocytes is not attributable to hydrogen peroxide but to the superoxide anion.

Fig 9 shows adenosine concentration of the effluent of polymorphonuclear leukocytes with and without a 30-minute exposure to 10⁻⁶ M FMLP. Adenosine release
from the polymorphonuclear leukocytes was higher in the untreated condition compared with the FMLP-pre- treated condition (Fig 9, A). This attenuation of adenosine release agrees well with the decreased ecto-5'-nucleotidase activity in polymorphonuclear leukocytes. Furthermore, when the reduction of ecto-5'-nucleotidase was restored by administration of SOD, capability of adenosine release in polymorphonuclear leukocytes was also restored to the levels of the untreated control condition (Fig 9, B). Adenosine release was increased in the hypoxic polymorphonuclear leukocytes; however, increases in adenosine release in the FMLP-pre-treated cardiomyocytes were lower compared with the untreated hypoxic condition (Fig 10, A). Concomitant administration of SOD restored the capability of adenosine release in the FMLP-pre-treated polymorphonuclear leukocytes in the hypoxic condition to the levels of the untreated hypoxic condition (Fig 10, B). Fig 11 shows the viability of polymorphonuclear leukocytes pretreated with FMLP and complement C5a with and without SOD. Both FMLP and complement C5a did not change the cellular viability in the control condition and slightly reduced the cellular viability in the hypoxic condition. At 60 minutes of reoxygenation, the viability of polymorphonuclear leukocytes was markedly reduced in the FMLP-pre-treated and complement C5a-pretreated groups. When SOD was concomitantly pretreated in the presence of FMLP and complement C5a, the cellular viability was restored to the control level. The Table shows the shape changes of polymorphonuclear leukocytes due to exposures to FMLP and complement C5a with and without SOD. Either exposure to FMLP or complement C5a caused the nonspherical changes of polymorphonuclear leukocytes, and this change was prevented by a concomitant exposure to SOD. Ten minutes after washout of FMLP or complement C5a, the cell shapes returned spherical. During hypoxia and reperfusion, nonspherical shape changes of polymorphonuclear leukocytes occurred in the FMLP-pre-treated and complement C5a-pretreated groups; these changes were prevented by concomitant pretreatment with SOD. These results indicate that deactivation of ecto-5'-nucleotidase and atten-
Reduction of Ecto-5'-nucleotidase Activity as a Potential Cause of Decreased Adenosine Release in Polymorphonuclear Leukocytes

We have shown that adenosine release from polymorphonuclear leukocytes is attenuated by exposures to FMLP and complement C5a in normoxic and hypoxic conditions through attenuation of ecto-5'-nucleotidase activity. We need to consider the cause-effect relation between the reduction of ecto-5'-nucleotidase activity and adenosine release in polymorphonuclear leukocytes. The major pathways of adenosine formation are the enzymatic dephosphorylation of 5'-adenosine monophosphate by 5'-nucleotidase and the hydrolysis of 5'-adenosylhomocysteine (SAH) by SAH-hydrolase. In the myocardium, Llyod and Schrader revealed that adenosine production through SAH-hydrolase is essential. However, in polymorphonuclear leukocytes, the content of SAH is reported to be considerably small to produce adenosine, suggesting that adenosine production through 5'-nucleotidase is more likely. 5'-Nucleotidase is membrane bound (ecto-5'-nucleotidase) and free in the cytoplasm (cytosolic 5'-nucleotidase), both having a capacity to produce adenosine. However, the question remains as to which is more important, ecto-5'-nucleotidase or cytosolic 5'-nucleotidase, for adenosine release in polymorphonuclear leukocytes. Van Waeg and Van den Berge reported that α,β-methyleneadenosine 5'-diphosphate, an inhibitor of ecto-5'-nucleotidase, potently attenuates adenosine release from polymorphonuclear leukocytes, indicating that ecto-5'-nucleotidase plays an important role for production of adenosine. The present study also demonstrated that adenosine release was markedly reduced when ecto-5'-nucleotidase activity was reduced and cytosolic 5'-nucleotidase activity was unaltered. These results suggest that ecto-5'-nucleotidase activity may be important for the production of adenosine in polymorphonuclear leukocytes. However, the present study does not deny the possible role of cytosolic 5'-nucleotidase activity for the production of adenosine in the polymorphonuclear leukocytes. Several lines of evidence demonstrate that cytosolic 5'-nucleotidase is also essential for the production of adenosine in hypoxic polymorphonuclear leukocytes and cardiomyocytes. Taken together, both ecto-5'-nucleotidase and cytosolic 5'-nucleotidase synergistically contribute to adenosine production in polymorphonuclear leukocytes, and the present study has revealed that activation of polymorphonuclear leukocytes inactivates ecto-5'-nucleotidase and thereby attenuates adenosine release.

In addition to 5'-nucleotidase, we need to consider the activities of adenosine kinase and adenosine deaminase as potential determinants of adenosine production in polymorphonuclear leukocytes. Indeed, Schrader et al reported that adenosine kinase contributes to adenosine production in the cardiomyocytes. Furthermore, Van Waeg and Van den Berge have reported that adenosine deaminase is inactivated when polymorphonuclear leukocytes are activated by phorbol myristate acetate. They also showed that adenosine accumulation is increased when polymorphonuclear leukocytes are treated by phorbol myristate acetate and the adenosine accumulation is blunted by 2'-deoxycoformycin, an inhibitor of adenosine deaminase. In the present study,

**Discussion**

In the present study, we conclude that (1) activation of polymorphonuclear leukocytes attenuates their ecto-5'-nucleotidase activity and thereby attenuates adenosine release, (2) reduction of ecto-5'-nucleotidase activity is attributable to the generated superoxide anion in polymorphonuclear leukocytes, and (3) these reductions of ecto-5'-nucleotidase activity and adenosine release in activated polymorphonuclear leukocytes may contribute to the acceleration of cellular injury of activated polymorphonuclear leukocytes during hypoxia and reoxygenation processes.

**Fig 11.** Cellular viability of polymorphonuclear leukocytes activated by $10^{-6}$ M N-formyl-methionyl-leucyl-phenylalanine (FMLP) and complement C5a (5.0 x 10^{-7} g/mL) with and without concomitant exposures to superoxide dismutase (SOD). After the incubations with FMLP and complement C5a in the presence or absence of SOD, the polymorphonuclear leukocytes were washed twice (control) and then exposed to 60 minutes of hypoxia (hypoxia) followed by 60 minutes of reoxygenation (reoxygenation). At the end of hypoxia, cellular viabilities of polymorphonuclear leukocytes pretreated with FMLP and complement C5a were slightly reduced; viabilities were restored by SOD pretreatment. Cellular viabilities of polymorphonuclear leukocytes pretreated with FMLP and complement C5a were decreased during reoxygenation; however, concomitant SOD pretreatment restored (P < .001) the cellular viability (n=5 in each group). A post hoc Scheffe’s test was used to determine the significance level.
since we used 2'-deoxycoformycin and 5'-iodotubercidin to inhibit adenosine deaminase and adenosine kinase, attenuation of these two enzymes may not contribute to the attenuation of adenosine release in the activated polymorphonuclear leukocytes. Further investigation is necessary to determine how much effect adenosine kinase and adenosine deaminase possess to modify the amount of endogenous adenosine produced by 5'-nucleotidase in activated or inactivated polymorphonuclear leukocytes.

Effects of Deactivation of Ecto-5'-nucleotidase and Reduction of Adenosine on Cellular Injury of Activated Polymorphonuclear Leukocytes

In the present study, we showed that exposures to both FMLP and complement C5a decrease ecto-5'-nucleotidase activity of polymorphonuclear leukocytes through generated superoxide anion (Figs 5 through 8). When ecto-5'-nucleotidase activity of polymorphonuclear leukocytes was attenuated because of exposures to FMLP and complement C5a, adenosine release during hypoxia was reduced, and thereby viability of polymorphonuclear leukocytes was markedly reduced during reoxygenation following hypoxia. How does preservation of adenosine release in polymorphonuclear leukocytes relate to the preservation of cellular viability? During hypoxia, degradative substances, eg, lactate, are accumulated, which may cause substantial cellular acidosis. Upon reoxygenation, Ca\(^{2+}\) overload in polymorphonuclear leukocytes may be induced: Accumulated H\(^+\) during hypoxia is extruded via Na\(^+\)/H\(^+\) exchange with accumulation of Na\(^+\), and Na\(^+\)/Ca\(^{2+}\) exchange reversely acts to extrude Na\(^+\) and increase intracellular Ca\(^{2+}\). Since Ca\(^{2+}\) overload is known to cause cellular injury\(^{29}\) and adenosine is reported to attenuate cellular Ca\(^{2+}\) overload during ischemia, possibly via inhibition of the reverse Na\(^+\)/Ca\(^{2+}\) exchange\(^{50}\) and Ca\(^{2+}\) channels,\(^{10}\) the attenuation of Ca\(^{2+}\) overload during hypoxia and reoxygenation due to adenosine may preserve the cellular viability. Another possibility is that preservation of ecto-5'-nucleotidase activity of activated polymorphonuclear leukocytes due to SOD pretreatment increases adenosine release during hypoxia and reoxygenation, which attenuates the generation of superoxide anions\(^{53}\) and thereby preserves cellular viability. Although we could not clarify the subcellular mechanisms whereby adenosine preserves the viability of polymorphonuclear leukocytes upon reoxygenation following hypoxia, we suggest that the preservation of ecto-5'-nucleotidase activity protects cellular functions of polymorphonuclear leukocytes by the preservation of adenosine release.

Pathophysiological Relevance

Adenosine is reported to attenuate ischemia and reperfusion injuries\(^{3-13}\) via its various cardiovascular actions.\(^{4-12}\) There are several lines of evidence suggesting that adenosine A\(_1\) receptor stimulation in polymorphonuclear leukocytes attenuates their generation of superoxide anions.\(^{5,9}\) This finding may partially explain the beneficial roles of adenosine in ischemia and reperfusion injuries,\(^{13-17}\) although conflicting evidence against the beneficial effects of adenosine have also been reported.\(^{41,42}\) Since adenosine is also produced in polymorphonuclear leukocytes, released adenosine seems to be the most important autocrine regulator for the function of polymorphonuclear leukocytes. Activated polymorphonuclear leukocytes are tightly related to the cause of reperfusion injury,\(^{16-20}\) which may injure myocardial cells as well as polymorphonuclear leukocytes themselves. We used different kinds of chemicals to activate polymorphonuclear leukocytes, FMLP, and complement C5a. Since FMLP is a pharmacological chemical activator and FMLP exposure may be different from the realistic situation for activation of polymorphonuclear leukocytes during reperfusion followed by ischemia, we have also used complement C5a and have obtained comparable results. Indeed, complement C5a is reported to be produced during ischemia and reperfusion via activation of polymorphonuclear leukocytes.\(^{43}\) These results suggest that superoxide anion–induced decreases in ecto-5'-nucleotidase in the activated polymorphonuclear leukocytes may play an important pathophysiological role for ischemia and reperfusion injuries, although further efforts are necessary.

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