The nondepolarizing, normokalemic cardioplegia formulation adenosine-lidocaine (adenocaine) exerts anti-neutrophil effects by synergistic actions of its components

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Objective: A new strategy of normothermic cardioplegia based on the combination of adenosine and lidocaine (adenocaine; Hibernation Therapeutics Global Ltd, Kilquade, Ireland) achieves nondepolarized arrest at normokalemia. Both adenosine and lidocaine independently inhibit neutrophil (polymorphonuclear neutrophil; PMN) activity. However, whether adenocaine exerts greater anti-inflammatory effects is not known. We tested the hypothesis that adenocaine synergistically attenuates PMN functions.

Methods: Superoxide anion (O_2^-) generation: Isolated porcine PMNs were primed with cytochalasin B (5 μ g/mL) and activated by N-formylmethionyl-leucyl-phenylalanine (100 nM). O_2^- release was quantified using lucigenin-enhanced chemiluminescence. Data were expressed as percent of stimulated control.

Results: Both adenosine and lidocaine alone inhibited O_2^- production in a dose-dependent manner (adenosine reduced to 67% $\pm$ 8.4% and 21% $\pm$ 2.2% of maximal stimulation at 0.1 and 10 μ mol/L, respectively, lidocaine reduced to 57.9% $\pm$ 18.6% and 28% $\pm$ 5% at 10 and 100 μ mol/L, respectively). Adenocaine further reduced O_2^- generation in a synergistic manner. In addition, adenosine alone (0.1–10 μ mol/L) inhibited O_2^- generation in primed but not activated PMNs, whereas lidocaine alone (1–100 μ mol/L) inhibited O_2^- release in both primed and activated PMNs. Adenocaine further reduced O_2^- generation because of inhibition of both priming and activation stages. Both adenosine and lidocaine alone and adenocaine comparably inhibited platelet activating factor-induced CD11 b/c surface expression on PMNs (flow cytometry), but adenocaine further suppressed both CD18 expression (to 47.4% $\pm$ 9.7%) and PMN adherence (to 47.2% $\pm$ 4.3%) compared with adenosine and lidocaine alone. Transmigration of calcein-acetoxymethyl-labeled PMNs through transwells seeded with cultured coronary artery endothelial cells was reduced comparably by adenosine (to 80.1% $\pm$ 6.7%) and adenocaine (67.3% $\pm$ 9.6%).

Conclusions: Adenocaine suppresses multiple PMN functions including O_2^- generation, adhesion molecule expression, PMN adherence, and transmigration. In addition to inducing nondepolarized arrest, adenocaine cardioplegia may exert cardioprotection by inhibiting PMN-mediated inflammatory responses. (J Thorac Cardiovasc Surg 2012;143:1167-75)

Hyperkalemic cardioplegia has been widely used in cardiac surgery where elective cardiac arrest is achieved by depolarizing the myocyte membrane. However, hyperkalemic cardioplegia has several complications. Membrane depolarization by hyperkalemia is associated with a persistent ion transmembrane flow through sodium and calcium “window currents,” causing intracellular sodium and calcium overload, energy exhaustion, arrhythmias, and cardiomyocyte and endothelial cell dysfunction and death.¹ In

inflammatory responses, the adherence of neutrophils to cardiomyocytes leads to depolarization of the myocyte, sodium influx, and cell death.² An alternative approach to achieving elective cardiac arrest is to maintain the transmembrane potential in a polarized state,^{3,4} which clamps the ion channels in a “closed” state and thereby prevents subsequent cellular depolarization and the formation of the action potential. Thus, maintaining cardiomyocytes in a polarized state may avoid window currents, ionic imbalances, and subsequent consequences. The combination of adenosine and lidocaine arrests the heart in a polarized state without hyperkalemia.^{5,6} Adenosine also stimulates adenosine triphosphate-sensitive potassium channels by activation of the A_1 receptor. Lidocaine inhibits transmembrane depolarization by blocking sodium fast channels. The combination of adenosine and lidocaine in cardioplegia (adenocaine; Hibernation Therapeutics Global Ltd, Kilquade, Ireland) has been shown to successfully induce polarized arrest in isolated rat hearts^{4,6} and in canine hearts on cardiopulmonary bypass when delivered

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Abbreviations and Acronyms

AM	=	acetyoxymethyl
Cyto B	=	cytochalasin B
FBS	=	fetal bovine serum
fMLP	=	formylmethionyl-leucyl-phenylalanine
HBSS	=	Hank's buffered salt solution
IL	=	interleukin
PAF	=	platelet activating factor
PCAEC	=	pig coronary arterial endothelial cell
PECGM	=	Porcine Endothelial Cell Growth Medium
PMN	=	polymorphonuclear neutrophil
RLU	=	relative light units
RPMI	=	Roswell Park Memorial Institute
SEM	=	standard error of the mean

normothermically or hypothermically.⁷ The concentrations of adenosine and lidocaine that achieve polarized arrest range from 200 and 500 $\mu\text{mol/L}$, respectively, in isolated hearts using a crystalloid solution,⁴ to 400 and 750 $\mu\text{mol/L}$, respectively, in blood cardioplegia.⁷ However, adenosine has also been used clinically at lower concentrations in moderately hyperkalemic blood cardioplegia in which the adenosine concentration is approximately 56 $\mu\text{mol/L}$ and lidocaine is approximately 64 $\mu\text{mol/L}$.⁸

The activation of polymorphonuclear neutrophils (PMNs) and the inflammatory response during cardiopulmonary bypass and cardioplegia likely contribute to post-cardioplegia dysfunction and adverse outcomes.⁹ Both adenosine¹⁰ and lidocaine¹¹ individually exert anti-inflammatory properties. Adenosine attenuates PMN proinflammatory functions and reperfusion injury mainly through activation of A_2 and possibly A_3 receptors.^{10,12,13} Lidocaine has also been shown to inhibit the inflammatory response to reperfusion injury, although the mechanism is unclear.¹⁴ This study tested the hypothesis that the combination of adenosine and lidocaine (adenocaine) inhibits inflammatory functions of PMNs. In particular, we determined the lowest concentrations of components in adenocaine that potentially exerted anti-PMN effects.

MATERIALS AND METHODS**Chemicals**

Chemicals used in this study were purchased from Sigma-Aldrich (St Louis, Mo) unless otherwise stated. All chemicals were prepared in double-distilled H_2O or in dimethyl sulfoxide. In cases in which dimethyl sulfoxide was used, its concentration was maintained at less than 0.1% in solutions.

Polymorphonuclear Neutrophil Isolation

All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (available at: <http://www.nap.edu/catalog/5140.html>).

PMNs were prepared using a protocol described previously.¹⁵ Briefly, peripheral blood (20 mL) was withdrawn from Yorkshire pigs using an arterial access and mixed with 10 mL 6% dextran and 4.5 mL anticoagulating buffer containing 1.6% citric acid and 2.5% sodium citrate. The blood sample was kept at room temperature for 40 minutes to sediment erythrocytes. The leukocyte-rich top layer was centrifuged at 400g for 10 minutes at 4°C. The pellet was lysed for 20 seconds with 9 mL of sterile distilled water to remove red blood cells, followed by the addition of 3 mL of 0.6 mol/L KCl and 25 mL of Hank's buffered salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Mediatech Inc, Manassas, Va). After another 10 minutes of centrifugation, the pellet was resuspended in 2 mL HBSS without Ca^{2+} and Mg^{2+} , layered on the top of 3 mL of Ficoll-Paque (GE Healthcare, Piscataway, NJ), and centrifuged at 400g for 30 minutes at 4°C. The cell pellet was resuspended in ice-cold HBSS without Ca^{2+} and Mg^{2+} . The cell number was counted. The final cell suspension contained 94% PMNs, and cell viability was 99% as determined by trypan blue exclusion. The PMNs were used within 4 hours after isolation.

Endothelial Cell Culture

Pig coronary arterial endothelial cells (PCAECs) used in adhesion and transmigration studies were purchased from Cell Applications Inc (San Diego, Calif). The cells were cultured as a monolayer in Porcine Endothelial Cell Growth Medium (PECGM; Cell Applications Inc) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 , and routinely split when cell density reached 90% to 100% confluence. The cells were used between passages 3 and 6.

Superoxide Production by Polymorphonuclear Neutrophils

PMNs were primed with cytochalasin B (Cyto B; 5 $\mu\text{g/mL}$, Sigma-Aldrich) for 5 minutes and activated by N-formylmethionyl-leucyl-phenylalanine (fMLP; 100 nM). The samples were placed in a luminometer (Autolumat Plus LB953; Berthold Tech, Bad Wildbad, Germany). Lucigenin (10 $\mu\text{mol/L}$) in HBSS was automatically injected into each assay tube. Photon emissions from the samples were counted sequentially under atmospheric conditions. After background readings were taken, the lucigenin-enhanced chemiluminescence reaction was read over 10 minutes, and the average background signal was subtracted. The superoxide anion (O_2^-) release was expressed as relative light units (RLU) per 10^6 PMNs ($\text{RLU}/10^6$).

Flow Cytometry

Because of limited quality and availability of antibodies against porcine adhesion molecules, heparinized rat blood drawn from the carotid artery was used in flow cytometry studies. Arterial blood was anticoagulated with heparin; pretreated with adenosine, lidocaine, or adenocaine at the doses indicated (see "Results") for 5 minutes; and stimulated by 100 nM platelet activating factor (PAF) for 15 minutes at room temperature. Then, 5 μL of phycoerythrin-conjugated anti-CD11 b/c (OX-42; BD Pharmingen, San Diego, Calif) or 5 μL of fluorescein isothiocyanate-conjugated anti-CD18 (WT.3; BD Pharmingen) was incubated with 50 μL of blood sample for 15 minutes at room temperature in the dark. Red blood cells were lysed for 10 minutes using 2 mL of FACSlyse (BD Pharmingen). After centrifugation at 400g for 5 minutes, cells were fixed with 1% paraformaldehyde and stored at 4°C. Data were acquired on a BD LSR II (BD Pharmingen). PMNs were identified by their forward scatter and side scatter profile using mouse anti-granulocyte antibody (His48; eBioscience Inc, San Diego, Calif). Autofluorescence was measured from cells stained with isotype control antibody. The expression of CD11b/c or CD18 was quantified using median fluorescence intensity with autofluorescence subtraction. The effect of adenosine, lidocaine, and adenocaine was calculated and normalized to a percent of basal expression (HBSS treated) versus the PAF-induced expression, and reported as means $\pm$ standard error of the mean (SEM).

Polymorphonuclear Neutrophil Labeling

Porcine PMNs ($1 \times 10^7/\text{mL}$) were resuspended in Roswell Park Memorial Institute (RPMI) 1640 (a type of leukocyte culture medium previously developed at Roswell Park Memorial Institute) containing 0.5% fetal bovine serum (FBS) and labeled with calcein-acetyoxymethyl (AM) ($1 \mu\text{mol/L}$; Molecular Probe, Carlsbad, Calif) in RPMI 1640 for 30 minutes at 37°C . The cell pellets were obtained by centrifugation ($400g$) for 5 minutes at 4°C and washed with phosphate-buffered saline twice. After counting, the PMNs were resuspended in RPMI 1640 with 0.5% FBS for adherence and transmigration studies (see below).

Polymorphonuclear Neutrophil Adherence Assay

PCAECs were cultured in a 96-well cell culture microplate (Corning Inc, Corning, NY) until reaching 90% to 95% confluence. The PECGM was gently exchanged for RPMI 1640 with 0.5% FBS for serum starvation before adherence experiments. Calcein AM-labeled PMNs ($5 \times 10^5/\text{well}$) were pretreated with given concentrations of adenosine, lidocaine, or adenocaine for 5 minutes and then stimulated by PAF (100 nM). Aliquots of PMN suspension were layered over the endothelial cells. After 15 minutes of incubation, the PMNs were gently aspirated from the endothelial cell monolayer. The microplate wells were carefully washed 3 times with prewarmed RPMI 1640 to remove nonadherent PMNs. An aliquot of PMNs was serially diluted, and fluorescence readings (absorbance of 494 nm and emission of 517 nm) were obtained from Spectra MAX Gemini EM (Molecular Devices, Sunnyvale, Calif). Standard curves were plotted using the cell concentrations against the fluorescence to quantify the number of cells adhered. The effect of adenosine, lidocaine, and adenocaine was measured and normalized between the basal adherence and the PAF-induced adherence, and expressed as means $\pm$ SEM.

Polymorphonuclear Neutrophil Transmigration Assay

Cell culture transwells ($3.0\text{-}\mu\text{m}$ pore-size insert, polyester membrane, 6.5 mm dimension, Costar; Corning Inc) were rinsed in PECGM for 30 minutes in an incubator before use. One day before the transmigration assay, PCAECs (2×10^5) were seeded onto each transwell insert. After overnight culture, the PCAECs formed a confluent monolayer. The culture medium was changed to RPMI 1640 with 0.5% FBS for serum starvation. Calcein-AM labeled 10^6 PMNs per well were added to the top chamber. Porcine interleukin (IL)-8 (10 nM ; R&D Systems, Minneapolis, Minn) was added to the lower chamber as chemoattractant. The transwell systems were incubated at 37°C for 1 hour, after which the inserts were removed and the fluorescence emitted from the lower chamber was measured using a Spectra MAX Gemini EM. The number of migrated PMNs was quantified using a similar protocol described in the adherence assay. IL-8-induced transmigration with the addition of vehicle, adenosine, lidocaine, or adenocaine was normalized against the basal transmigration.

Statistical Analysis

The effects of adenosine, lidocaine, or adenocaine (3–10 separate experiments in each assay) were expressed as a percent of the corresponding stimulated control. Values were presented as means $\pm$ SEM. Group differences were analyzed using a 1-way analysis of variance. Where group differences were detected, post hoc Tukey's or Student-Newman-Keuls multiple comparison tests were performed to determine which groups differed.

RESULTS

O_2^- Production

Cyto B is a widely used priming agent, whereas fMLP acts as an activator of PMNs.¹⁶ The resting PMNs demonstrated a low level of O_2^- generation of less than 10

FIGURE 1. O_2^- generation by cytochalasin B (Cyto B, $5 \mu\text{g/mL}$) and fMLP (100 nM). A, Time course of O_2^- production from PMNs under different stimulatory conditions. Cyto B or fMLP alone induced only a mild O_2^- release. The fully activated PMNs that were primed with Cyto B and subsequently activated by fMLP (Cyto B + fMLP) released O_2^- in a burst pattern. B, Summary of the maximal O_2^- production in the presence of different stimulators. The O_2^- generation was significantly enhanced in the Cyto B + fMLP group. $*P < .05$ versus Cyto B-primed and fMLP-activated PMNs. PMN, Polymorphonuclear neutrophil; RLU, relative light units; fMLP, formylmethionyl-leucyl-phenylalanine.

RLU/ 10^6 cells. The O_2^- generation induced by Cyto B or fMLP alone was modest with a peak averaging 52 ± 6 RLU/ 10^6 cells ($n = 8$) for Cyto B alone and 64 ± 14 RLU/ 10^6 cells ($n = 9$) for fMLP alone (Figure 1, A, B). In contrast, in the fully activated PMNs first primed by Cyto B ($5 \mu\text{g/mL}$) and subsequently activated by fMLP (100 nM), O_2^- was produced in a burst pattern (Figure 1, A) with a significantly higher peak O_2^- release averaging 207 ± 54 RLU/ 10^6 cells ($n = 17$, Figure 1, B).

The effect of adenosine and lidocaine was normalized to the maximal stimulation of PMNs by $5 \mu\text{g/mL}$ Cyto B plus 100 nM fMLP after correcting for blank (HBSS alone). Superoxide dismutase (0.1 mg/mL) almost completely abolished the O_2^- generation, confirming that the chemiluminescence signal was largely derived from O_2^- (Figure 2, A–C). Adenosine and lidocaine alone inhibited O_2^- production in a concentration-dependent manner (Figure 2, B, C); $1 \mu\text{mol/L}$ adenosine reduced the O_2^-

FIGURE 2. Effects of adenosine and lidocaine on O_2^- generation in fully activated PMNs. A, Time course of O_2^- generation from the fully activated PMNs. The untreated PMNs (HBSS) showed a small O_2^- generation. Adenosine (A) suppressed O_2^- production in a dose-dependent manner. Superoxide dismutase (0.1 mg/mL) almost abolished the O_2^- production. B, Concentration-dependent inhibition of O_2^- generation by adenosine. The effect of adenosine was measured and normalized between the basal O_2^- production from the untreated PMNs and the maximal O_2^- generation induced by Cyto B and fMLP (mean $\pm$ SEM). * $P < .05$ versus untreated PMNs. C, Lidocaine inhibited O_2^- production in a dose-dependent manner. * $P < .05$ versus untreated PMNs. D, Shown is a summary of the effects of adenosine and lidocaine on O_2^- production. Adenosine (1 or 10 μ mol/L) and lidocaine (10, 100, 1000 μ mol/L) reduced O_2^- generation. * $P < .05$ versus untreated PMNs. The addition of 1 μ mol/L adenosine to 100 or 1000 μ mol/L lidocaine further suppressed the O_2^- generation. + $P < .05$ vs adenosine (1 μ mol/L) group. In the presence of 10 μ mol/L lidocaine, adenosine (10 μ mol/L) provided further inhibition. † $P < .05$ vs lidocaine (10 μ mol/L) group. Cyto B, Cytochalasin B; HBSS, Hanks' buffered salt solution; fMLP, N-formylmethionyl-leucyl-phenylalanine; PMN, polymorphonuclear neutrophil; SOD, superoxide dismutase.

generation to $53.6\% \pm 12.5\%$ of stimulated control ($n = 3$). Lidocaine (10 μ mol/L, 0 μ mol/L adenosine) alone decreased O_2^- production to $57.9\% \pm 18.6\%$ of stimulated control (Figure 2, D; $n = 3$, $P < .05$). The addition of lidocaine (100 or 1000 μ mol/L) to 1 μ mol/L adenosine further significantly suppressed the O_2^- generation to $12.4\% \pm 2.9\%$ ($n = 3$) and $0.8\% \pm 1.4\%$ ($n = 3$), respectively, relative to 1 μ mol/L adenosine alone. With the combination of 10 μ mol/L adenosine and 10 μ mol/L lidocaine, the O_2^- release was further decreased to $18.3\% \pm 1.9\%$ ($n = 3$, $P < .05$). The greatest significant reduction in O_2^- release was observed with 10 μ mol/L adenosine and 100 μ mol/L lidocaine. Therefore, the combination of 10 μ mol/L adenosine and 100 μ mol/L lidocaine exhibited synergistic inhibition of O_2^- generation by activated PMNs.

To determine the individual effect of adenosine and lidocaine on O_2^- PMN in primed versus activated stages, PMNs were stimulated with Cyto B (priming) or fMLP (activation) separately. Adenosine demonstrated a concentration-response relationship. As low as 0.1 μ mol/L adenosine suppressed the Cyto B-induced O_2^- generation to $37.0\% \pm 13.2\%$ of stimulated control values (Figure 3, A; $n = 4$,

$P < .05$ vs Cyto B-treated PMNs). In contrast, there was no such concentration-response relationship demonstrated in activated only PMNs; a higher concentration of adenosine (100 μ mol/L) was required to significantly inhibit the fMLP-induced O_2^- production (Figure 3, A; $73.5\% \pm 11.4\%$, $n = 5$, $P < .05$ vs fMLP-treated PMNs). However, lidocaine attenuated O_2^- generation after both priming alone and activation alone in a concentration-response pattern (Figure 3, B). Clear inhibition in either group was obtained with 10 μ mol/L lidocaine ($69.3\% \pm 6.1\%$, $n = 4$, $P < .05$ vs Cyto B-treated PMNs and $55.2\% \pm 9.6\%$, $n = 4$, $P < .05$ vs fMLP-treated PMNs, respectively). To simplify the subsequent studies, 50 μ mol/L adenosine and 100 μ mol/L lidocaine were chosen because these concentrations were effective in inhibiting O_2^- generation in the present study and used in a previous in vivo study using a porcine model of left ventricular arrest and resuscitation.¹⁷ The Cyto B-induced O_2^- production was reduced to $21.7\% \pm 3.0\%$ by 50 μ mol/L adenosine and to $19.1\% \pm 3.7\%$ by 100 μ mol/L lidocaine (Figure 3, C; $n = 8$, $P < .05$ vs Cyto B-treated group). The combination of 50 μ mol/L adenosine and 100 μ mol/L lidocaine further decreased O_2^- production

FIGURE 3. Summary of effects of adenosine and lidocaine on O_2^- production by Cyto B and fMLP. A, Inhibition of the Cyto B-induced O_2^- generation was seen with 0.1 $\mu\text{mol/L}$ adenosine ($*P < .05$ vs Cyto B-treated PMNs), whereas 100 $\mu\text{mol/L}$ adenosine significantly inhibited the fMLP-induced O_2^- production ($+P < .05$ vs fMLP-treated PMNs). B, Lidocaine had a similar effect on O_2^- production induced by Cyto B or fMLP. A significant reduction of O_2^- release from Cyto B-treated or fMLP-treated PMNs was evident with more than 10 $\mu\text{mol/L}$ lidocaine, with an 80% reduction observed with 100 $\mu\text{mol/L}$ lidocaine ($*P < .05$ vs Cyto B-treated PMNs and $+P < .05$ vs fMLP-treated PMNs, respectively). C, Cyto B-induced O_2^- production was reduced to 21.7% by 50 $\mu\text{mol/L}$ adenosine (A50) and to 19.1% by 100 $\mu\text{mol/L}$ lidocaine (L100) ($n = 8$, $*P < .05$ vs Cyto B-treated group). The combination of 50 $\mu\text{mol/L}$ adenosine and 100 $\mu\text{mol/L}$ lidocaine (A50L100) further decreased O_2^- production to 11.0% ($n = 8$, $\dagger P < .05$ vs A50 or L100). D, O_2^- generation from activated PMNs (fMLP treated) was decreased to approximately 70% by A50 and approximately 35% by L100 ($n = 7$, $*P < .05$ vs fMLP-treated group), whereas it was decreased further to approximately 33% of stimulated controls with the combination of A50L100 ($n = 7$, $\dagger P < .05$ vs A50). fMLP, Formylmethionyl-leucyl-phenylalanine.

to $11.0\% \pm 2.1\%$ ($n = 8$, $P < .05$ vs 50 $\mu\text{mol/L}$ adenosine or 100 $\mu\text{mol/L}$ lidocaine). The fMLP-induced O_2^- generation was decreased to $71.0\% \pm 5.2\%$ by 50 $\mu\text{mol/L}$ adenosine and to $35.1\% \pm 3.7\%$ by 100 $\mu\text{mol/L}$ lidocaine (Figure 3, D; $n = 7$, $P < .05$ vs fMLP-treated group). The combination of 50 $\mu\text{mol/L}$ adenosine and 100 $\mu\text{mol/L}$ lidocaine decreased O_2^- production to $32.8\% \pm 5.1\%$ ($n = 7$, $P > .05$ vs 100 $\mu\text{mol/L}$ lidocaine, $P < .05$ vs 50 $\mu\text{mol/L}$ adenosine).

Polymorphonuclear Neutrophil CD11 and CD18 Expression

Ischemia-reperfusion injury elevates surface expression of β_2 integrins on PMNs. The heterodimeric β_2 integrins contain 2 subunits with an α subunit (CD11a, b, or c) and β subunit (CD18). By using a phycoerythrin-labeled anti-CD11 b/c and a fluorescein isothiocyanate-labeled anti-CD18 antibody, we quantified the median fluorescence intensity of PMNs. Fifteen-minute incubation with PAF (100 nM) in antibody-labeled PMNs increased the median fluorescence intensity of PMN CD11 b/c from 61.2 ± 8.9 to 94.2 ± 6.7 (Figure 4, A; $n = 7$, $P < .05$) and enhanced CD18 expression from 171.8 ± 5.2 to 216.2 ± 7.9 (Figure 4, B; $n = 7$, $P < .001$). Preincubation of the blood sample with 50 $\mu\text{mol/L}$ adenosine (Figure 4, C) before

PAF activation significantly reduced the CD11 b/c expression to $59.0\% \pm 8.2\%$ of control compared with PAF-activated PMN ($n = 7$, $P < .05$). Lidocaine at 100 $\mu\text{mol/L}$ decreased the CD11 b/c expression to $71.4\% \pm 6.3\%$ (Figure 4, C; $n = 7$, $P < .05$) of stimulation control. The combination of adenosine and lidocaine at 50 and 100 $\mu\text{mol/L}$, respectively, tended to further reduce CD11 b/c expression to $48.2\% \pm 10.3\%$ of controls (Figure 4, C; $n = 7$, $P < .05$), but this was not significant relative to the 50 $\mu\text{mol/L}$ adenosine or the 100 $\mu\text{mol/L}$ lidocaine group. Likewise, 50 $\mu\text{mol/L}$ adenosine significantly inhibited the CD18 expression to $69.5\% \pm 9.4\%$ compared with PAF-activated PMN (Figure 4, D; $n = 7$, $P < .05$), whereas 100 $\mu\text{mol/L}$ lidocaine reduced the CD18 expression to $81.0\% \pm 8.6\%$ (Figure 4, D; $n = 7$, $P < .05$) of stimulation control. However, the combination of adenosine (50 $\mu\text{mol/L}$) and lidocaine (100 $\mu\text{mol/L}$) significantly further decreased the CD18 expression to $47.4\% \pm 9.7\%$ (Figure 4, D; $n = 7$, $P < .05$) relative to identical concentrations of adenosine or lidocaine alone.

Polymorphonuclear Neutrophil Adherence

When unstimulated PMNs were added to the 96-well plate and incubated with endothelial cells for 15 minutes,

FIGURE 4. Decreased CD11/18 surface expression on PMNs by adenosine and lidocaine. A and B, The surface expressions of PMN CD11 b/c and CD18 were enhanced by PAF (100 nM) (+ $P < .05$, +++ $P < .001$). C, Pretreatment of blood with 50 $\mu\text{mol/L}$ adenosine (A50) before PAF stimulation significantly reduced the CD11 b/c expression compared with PAF-activated PMN ($n = 7$, * $P < .05$). Lidocaine at a concentration of 100 $\mu\text{mol/L}$ (L100) decreased the CD11 b/c expression to $71.4\% \pm 6.3\%$ ($n = 7$, * $P < .05$) of stimulation control. The combination of 50 $\mu\text{mol/L}$ adenosine and 100 $\mu\text{mol/L}$ lidocaine (A50L100) further reduced the CD11 b/c expression ($n = 7$, * $P < .05$), but the effect was not significant from adenosine or lidocaine alone. D, Both A50 and L100 significantly reduced the CD18 expression compared with PAF-activated PMN ($n = 7$ each, * $P < .05$). A50L100 further decreased CD18 expression ($n = 7$, * $P < .05$), and the effect was significantly different from both adenosine and lidocaine ($n = 7$, † $P < .05$). MFI, Median fluorescence intensity; PAF, platelet activating factor; HBSS, Hank's buffered salt solution.

an average of 3.9×10^4 cells were observed to adhere to the endothelial surface. Activation of PMNs with 100 nM PAF resulted in a near doubling of adherence to 7.5×10^4 cells (Figure 5, A; $n = 19$, $P < .001$). Both adenosine (Figure 5, B) and lidocaine (Figure 5, C) alone inhibited PAF-induced PMN adherence to endothelial cells. As shown in Figure 5, D, preincubation of PMNs with 50 $\mu\text{mol/L}$ adenosine before PAF activation significantly reduced the adherence to $71.6\% \pm 5.4\%$ compared with PAF-activated PMN ($n = 8$, $P < .05$), whereas 100 $\mu\text{mol/L}$ lidocaine alone decreased the adherence to $73.2\% \pm 8.1\%$ (Figure 5, D; $n = 7$, $P < .05$) of stimulation control. The combination of adenosine and lidocaine significantly further reduced the adherence to $47.2\% \pm 4.3\%$ (Figure 5, D; $n = 7$, $P < .05$).

Adenosine Inhibited Polymorphonuclear Neutrophil Transmigration Across Coronary Artery Endothelial Cells

We tested the migration of PMN through PCAECs towards the chemoattractant IL-8 contained in the lower chamber of the transwell. In the presence of IL-8 (10 nM), the average number of PMNs that migrated across the endothelial cell layer increased from 16×10^4 (no IL-8) to 52×10^4 (Figure 6, A; $n = 6$, $P < .05$). After pretreatment

with adenosine (50 $\mu\text{mol/L}$), transendothelial migration of PMNs was reduced to $80.1\% \pm 6.7\%$ of control PCAECs (Figure 6, B; $n = 6$, $P < .05$). Lidocaine (100 $\mu\text{mol/L}$) did not significantly decrease the transmigration of PMNs (Figure 6, B; $82.9\% \pm 17.0\%$, $n = 6$, $P > .05$) due in part to the variability around the mean value. The combination of 50 $\mu\text{mol/L}$ adenosine and 100 $\mu\text{mol/L}$ lidocaine did not significantly further reduce transmigration, although a small trend was observed (Figure 6, B; $67.3\% \pm 9.6\%$, $n = 6$, $P > .05$).

DISCUSSION

The results of the present study demonstrate the following: (1) Adenosine alone inhibits PMN O_2^- generation, CD11 and CD18 surface expression, adherence, and transmigration in vitro. In addition, adenosine decreases superoxide production likely through inhibiting the priming process, whereas lidocaine inhibits both priming and activation. (2) Lidocaine alone did not significantly reduce PMN transmigration because of a larger variability around the average, whereas adenosine significantly inhibited transmigration. (3) Adenosine exerts greater inhibition of PMN O_2^- generation, CD18 surface expression, and adherence to endothelial cells when compared with adenosine or lidocaine alone. Adenosine inhibited O_2^- in both primed and

FIGURE 5. Reduced PMN adherence by adenosine and lidocaine. A, Calcein AM-labeled PMNs (5×10^5 /well) were layered over the confluent PCAECs. PAF (100 nM) increased adherence of PMNs ($n = 19$, $+++P < .001$). B, Pre-exposure of PMNs to adenosine reduced adherence. The effect of adenosine was measured and normalized between the basal adherence and the PAF-induced (100 nM) adherence, and described as mean $\pm$ SEM. Maximal activation was reached at 100 μ mol/L with no change thereafter. $*P < .05$ versus PAF-induced PMN adherence. C, Inhibition of PMN adherence with lidocaine was observed at 1 μ mol/L, with modest inhibition thereafter ($n = 6$, $*P < .05$ vs PAF-induced PMN adherence). D, The combination of adenosine (50 μ mol/L, A50) and lidocaine (100 μ mol/L, L100) additively inhibited PMN adherence. Adenosine and lidocaine both reduced PMN adherence, but adherence was further reduced by the combination of adenosine and lidocaine (A50L100) ($n = 7$, $*P < .05$ vs PAF-treated PMNs, $\dagger P < .05$ vs A50 or L100-pretreated PMNs). PAF, Platelet activating factor; PMN, polymorphonuclear neutrophil.

fully activated PMNs. The lowest effective concentrations of adenosine averaged 50 μ mol/L and lowest effective concentration of lidocaine averaged 100 μ mol/L. These concentrations are lower than the concentrations of adenosine and lidocaine that have been used in experimental studies used in isolated rat hearts⁴ and canine cardiopulmonary bypass model,⁷ and are similar to concentrations used in a clinical study in which adenosine was used in nonarresting concentrations in moderately hyperkalemic blood cardioplegia.⁸ Thus, adenosine suppresses PMN activation and other functions to a greater extent than each component drug alone. The beneficial effects reported by O'Rullivan and colleagues⁸ in a high-risk patient may, in part, be derived from inhibition of inflammatory responses to cardiopulmonary bypass.

Adenosine and lidocaine alone have been shown to exert anti-inflammatory effects. Adenosine inhibits PMN activation through A_{2a} receptors. A_{2a} receptor stimulation attenuates PMN-induced coronary endothelial injury by reducing O_2^- generation and adherence.¹⁵ In addition to blocking Na^+ fast channels, lidocaine also affects K^+ channels (G protein-gated inwardly rectifying K^+ channels and 2-pore domain K^+ channels^{18,19}) and vanilloid receptor TRPV1 channels. In addition to ion channel modulation, recent studies show that lidocaine can inhibit inflammation. As shown in the present study, lidocaine suppresses the generation of

oxygen free radical species²⁰ and attenuates the release of proinflammatory cytokines (IL-1 β , IL-8).²¹ Lidocaine has also been reported to reduce leukocyte adhesion²² and transmigration,²³ again consistent with the results of the present study. Some of these effects are not due to Na^+ channel inhibition, because the EC_{50} of certain effects are in the nanomolar range, which is less than the EC_{50} for Na^+ channel blockade (50–100 μ mol/L).¹¹ The mechanisms underlying these anti-inflammatory characteristics are not yet well understood. Lidocaine inhibits lipopolysaccharide-induced priming and NADPH oxidase assembly through suppression of cytochrome b558.²⁴ Another study suggests that lidocaine can reduce O_2^- generation through inhibiting p47 translocation.²⁰

O_2^- generation was induced by stimulating PMNs with Cyto B (primer) and fMLP (activator). Cyto B is a cytoskeleton disassembling toxin that increases the PMN response to other stimuli, such as PAF and fMLP,²⁵ and is widely used as a priming agent. The bacterial peptide fMLP is a strong leukocyte chemoattractant and activator agent. Cyto B or fMLP alone induced a small O_2^- production, which is in agreement with previous studies showing that full activation of PMN requires both priming and activating processes.¹⁶ The sequential use of Cyto B and fMLP simulated the priming that occurs with some cytokines, and subsequent activation by interaction with the vascular

FIGURE 6. Reduced PMN transmigration by adenosine. A, IL-8 (10 nM) increased the number of migrated PMNs ($n = 6$, $\dagger P < .05$ vs untreated PMNs). B, The effect of adenosine and lidocaine on transmigration. Data were normalized between the basal migration and the IL-8–induced migration. Adenosine (50 $\mu\text{mol/L}$, A50) significantly reduced the migration ($n = 6$, $*P < .05$ vs IL-8–treated group), whereas lidocaine (100 $\mu\text{mol/L}$, L100) only tended to inhibit migration ($P > .05$). The combination of adenosine and lidocaine (A50L100) did not significantly reduce transmigration compared with the adenosine alone group. IL, Interleukin; PMN, polymorphonuclear neutrophil.

endothelium. In the present study, we primed the isolated PMNs with Cyto B and then activated with fMLP to induce a peak oxidative burst as would be found in vivo. We found that both adenosine and lidocaine alone inhibited Cyto B–primed and fMLP-activated O_2^- generation. The combination of adenosine and lidocaine in adenocaine provided further inhibition compared with adenosine or lidocaine alone. This finding suggests that adenosine and lidocaine work in a synergistic manner in adenocaine.

Adenosine has been reported to diminish the priming of human PMN induced by PAF rather than tumor necrosis factor- α .²⁶ An interesting finding in our study is that adenosine as low as 0.1 $\mu\text{mol/L}$ significantly inhibited Cyto B–induced O_2^- generation, but only at a high concentration (100 $\mu\text{mol/L}$) can adenosine significantly reduce fMLP-induced O_2^- production. The different responses indicate that adenosine may predominantly target certain types of priming processes. The priming of PMNs can

also be blocked by lidocaine. Pretreatment of PMNs with lidocaine inhibits priming induced by lipopolysaccharide, PAF, or lysophosphatidic acid.^{23,24,27} In our study, lidocaine reduced the stimulatory effects of Cyto B and fMLP in a concentration-response pattern, suggesting lidocaine may target both priming and activating processes. Adenocaine effectively suppressed the effect of both priming (Cyto B) and activation (fMLP), which may reflect the predominance of lidocaine on both processes.

Adhesion molecule expression is one of the principle consequences of PMN priming¹⁶ and is another important event occurring in the early phase of reperfusion injury.⁹ Our data show that PAF-induced CD11 and CD18 surface expression was inhibited by adenosine and lidocaine alone, but that adenocaine further suppressed CD18 expression.

Vascular endothelial cells are key players in the inflammatory response to ischemia-reperfusion.²⁸ The localization and recruitment of blood leukocytes to inflammatory tissues are endothelial-dependent events and critical responses to inflammation.²⁸ Adenosine reduces the adherence of fMLP-stimulated PMN on cultured human umbilical vein endothelial cells²⁹ and PAF-induced adherence of PMN to endothelial layer of isolated canine coronary artery segments.¹⁵ Lidocaine at plasma concentrations in the range of clinical practice reduces leukocyte adhesion to artificial materials³⁰ and blood vessels.²² Our results are consistent with these previous studies. However, the combination of adenosine and lidocaine in adenocaine at the concentrations tested further decreased PAF-induced PMN CD18 expression and adherence, suggesting multifocal mechanisms in its anti-inflammatory effects.

The migration of PMN across endothelial layer is a critical event that occurs during ischemia-reperfusion injury. Adenosine diminishes ischemia-reperfusion–induced leukocyte extravasation in small intestine.³¹ We demonstrated that adenosine A_2 receptor activation significantly decreases PMN accumulation in the non-necrotic area at risk tissue in heart.¹³ In agreement, the current study suggests that adenosine decreased IL-8–induced PMN migration across the cultured coronary endothelial cells. Lidocaine reduces leukocyte migration in many in vivo studies.^{22,32} In our study, although lidocaine tended to attenuate PMN transmigration, the effect is not significant. In agreement, adenocaine did not provide further inhibition compared with adenosine alone.

CONCLUSIONS

Compared with adenosine and lidocaine alone, adenocaine exerts greater inhibitory effects on PMN activities by reducing O_2^- production, CD18 surface expression, and PMN adherence to vascular endothelium to a greater extent. These inhibitory effects can be exerted by adenocaine at lower concentrations of either component drug alone. Transmigration is also inhibited by adenocaine,

although the effect is not significantly greater than that of adenosine alone. The concentrations that exerted anti-inflammatory effects in the present study were similar to those used in a clinical study on adenocaine in cardiac surgery⁸ but lower than those proposed to arrest the heart in a polarized state without hyperkalemia.^{4,6} This lower, nonarresting dose of adenocaine may be effective in reducing the inflammatory responses to cardiopulmonary bypass and ischemia-reperfusion in moderately hyperkalemic cardioplegia. Lidocaine as a local anesthetic has longer lasting effects on Na⁺ channels and cellular membrane excitability, whereas adenosine has a short half-life in vivo because of rapid deamination by plasma adenosine deaminase. The longer-lasting effects of lidocaine might contribute to the synergistic actions of adenocaine over adenosine alone. The cardioprotection afforded by adenocaine may go beyond the advantages of polarized arrest on reducing oxygen demands and stabilizing electrical excitability to include exerting anti-PMN effects at relatively low, nonarresting concentrations, which may be important in attenuating the inflammatory response to both ischemia-reperfusion and cardiopulmonary bypass. Future studies are required to determine whether adenocaine exerts anti-PMN and other anti-inflammatory effects in clinical cardioplegia, and if such effects are exerted, whether these effects translated to better clinical outcomes.

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