Direct Treatment of Mouse or Human Blood With Soluble 5′-Nucleotidase Inhibits Platelet Aggregation

Melanie L. Hart, David Köhler, Tobias Eckle, Doris Kloor, Gregory L. Stahl, Holger K. Eltzschig

Objective—Adenosine signaling is known to inhibit platelet aggregation. Extracellular adenosine mainly stems from enzymatic phosphohydrolysis of precursor nucleotides via ecto-5′-nucleotidase. Previous studies suggest that soluble 5′-nucleotidase (5′-NT) derived from *Crotalus atrox* venom may be clinically beneficial in vascular leakage, myocardial, renal, and intestinal ischemia, or acute lung injury. However, the effects of 5′-NT treatment on platelet aggregation remain unknown. We examined the direct effects of 5′-NT treatment on platelet aggregation in vivo and ex vivo using a whole blood aggregation method.

Methods and Results—Platelet aggregation in whole human blood was completely inhibited by 5′-NT. When 5′-[$\alpha\beta$-methylene] diphosphate (APCP), a specific 5′-ecto-nucleotidase inhibitor, was added together with 5′-NT, APCP fully restored collagen- or ADP-induced aggregation. Adenosine levels in whole blood were significantly increased after 5′-NT treatment compared to controls and inhibition of platelet aggregation by 5′-NT was completely reversed by pretreatment with the nonspecific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline hydrate (8-SPT), suggesting that 5′-NT inhibits aggregation via increased adenosine signaling. Administration of 5′-NT to mice in vivo abolished ADP- and collagen-induced platelet aggregation and increased adenosine concentrations and tail bleeding time.

Conclusions—5′-NT treatment inhibits platelet aggregation via generation of increased levels of extracellular adenosine and subsequent adenosine receptor signaling. (***Arterioscler Thromb Vasc Biol*. 2008;28:1477-1483)**

Key Words: 5′-nucleotidase ■ platelets ■ aggregation ■ thrombosis ■ adenosine

Ecto-5′-nucleotidase (ecto-5′-nt, CD73) is a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule with ecto-enzymatic activity.[1] It is abundantly expressed on the vascular endothelium and catalyzes the extracellular conversion of AMP to adenosine,[2] a purine nucleoside implicated in many physiological events. Thus, CD73 is the final step of the extracellular nucleotide breakdown cascade that also involves membrane-associated ecto-ADPase (CD39; converts ATP/ADP to AMP).[3] Adenosine produced by CD73 can signal through 4 adenosine receptors designated A1, A2a, A2b, or A3.[4]

Hypoxia occurs when the maximum capacity for tissue oxygen extraction is exceeded and can result in clinical conditions such as adult respiratory distress syndrome (ARDS), sepsis, multiple trauma, severe liver failure, or severe pancreatitis. Ischemia/reperfusion injury (IRI) refers to organ dysfunction induced by constriction/obstruction of blood vessels that supply a particular organ. Many groups have shown that ecto-5′-nucleotidase (CD73) is protective during IRI or hypoxia and demonstrate that treatment with soluble 5′-nucleotidase (5′-NT) purified from *Crotalus atrox* snake venom prevents injury.[5–8] For example, CD73-deficient ($CD73^{-/-}$) mice demonstrated increased vascular leakage and pulmonary edema on hypoxia exposure.[7] Moreover, treatment with 5′-NT dramatically prevented vascular leakage in the colon, lung, liver, muscle, heart, and kidney. $CD73^{-/-}$ mice were also more susceptible to myocardial ischemia, and treatment of wild-type (WT) mice with 5′-NT significantly attenuated myocardial infarct size, suggesting use of 5′-NT for treatment of coronary artery disease.[6] Similarly, 5′-NT has been suggested as a therapeutic for renal diseases precipitated by limited oxygen availability such as surgical procedures requiring cross-clamping of the aorta and renal vessels (eg, during aneurysm repair or surgery for peripheral vascular disease), renal transplantation, or after cardiac surgery.[5,9] Thus, pharmacological inhibition of CD73 or its targeted gene deletion abolished renal protection by ischemic preconditioning (IP).[5] Treatment of $CD73^{-/-}$ mice with 5′-NT resulted in complete restoration of renal protection by IP, whereas injury after ischemia was significantly

Original received December 2, 2007; final version accepted May 17, 2008.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.108.169219**

attenuated in WT mice. Furthermore, treatment of CD73$^{-/-}$ or WT mice with 5′-NT significantly decreased intestinal IRI which can occur during strangulation-obstruction of the intestine, small bowel transplantation, cardiopulmonary bypass, and vascular or abdominal aortic surgery.[8] The importance of ecto-5′-nt has additionally been implicated in the resolution of inflammation during vascular remodeling[10,11] and bleomycin-[12] or ventilator-induced[13] lung injury. Together these results suggest that 5′-NT represents a potential therapeutic for a broad range of clinical conditions.

Platelets play a critical role in hemostasis and blood clotting at sites of vascular injury and therefore are important in the development of thrombosis. Antiplatelet therapy has become a mainstay in treatment or prophylaxis for conditions such as stroke, myocardial infarction, and other cardiovascular diseases. In recent years much attention has focused on inhibiting platelet aggregation to prevent thrombotic occlusion. Acetyl-salicylic acid (aspirin) is still the most widely used agent and considered the prototype antiplatelet drug. However, because platelet activation occurs via several pathways that are not influenced by aspirin, a number of compounds have been developed to complement the beneficial effect of aspirin. Currently, many agents are available but there is room for improvement via development of additional more effective agents.

It has been postulated for quite some time that adenosine formed at the endothelial/platelet interface may limit thrombus formation.[14–17] Because ecto-5′-nt represents the final step in extracellular adenosine production, it is an attractive target for understanding thrombus formation and has recently been shown to be involved in thromboregulation. Thus, it was demonstrated that ecto-5′-nt found on the surface of endothelial cells converts AMP to adenosine and inhibits platelet aggregation in vitro.[18] Additionally, CD73$^{-/-}$ mice subjected to free radical injury demonstrated faster coronary artery occlusion compared to WT mice.[10] Based on these studies and recent studies suggesting the novel use of 5′-NT for a variety of clinical conditions, it is critical to fully understand the effects of 5′-NT on platelet aggregation before use in the clinical setting. In the present study we examined the direct effects of 5′-NT on platelet aggregation in vivo and ex vivo using a whole blood aggregation method.

Methods

Mice

Experiments using A2a$^{+/+}$, A2a$^{-/-}$,[19] or C57BL/6 mouse blood were conducted under approved protocols (please see supplemental materials, available online at http://atvb.ahajournals.org). C57BL/6 mice were treated intraperitoneally (i.p.) with 500U/kg 5′-NT with or without 40 mg/kg 8-(p-sulfophenyl)theophylline hydrate (8-SPT, Sigma), a nonspecific adenosine receptor (AR) antagonist,[20] or saline. After 30 minutes, mice were anesthetized with sodium pentobarbital (70 mg/kg) and blood was drawn via cardiac puncture.

Whole Blood Aggregation

Whole blood aggregation studies were completed on the 560VS aggregometer (Chronolog) within 3 hours of collection. The protocol for blood donations was approved by the institutional review board and written informed consent was obtained from each individual prior to donation. Blood from healthy human volunteers or mice was drawn into a 0.1 volume of sodium citrate (3.8%), diluted 1:2 with saline and preincubated at 37°C for 5 minutes in an aggregometer cuvette (Chronolog). Platelet activation was initiated by the addition of 5 μg/mL collagen, 10 μmol/L ADP, 50 μmol/L epinephrine, 0.5 μmol/L arachidonic acid, or 0.1U/mL thrombin (Chronolog), and percent aggregation was measured for 6 minutes. In some experiments, whole blood was treated with 5U/mL 5′-NT purified from *Crotalus atrox* venom (Sigma) with or without 500 μmol/L 5′-[αβ-methylene] diphosphate (APCP, Sigma), a specific inhibitor of ecto-5′-NT[21]; 200 μmol/L 8-(p-sulfophenyl) the ophylline hydrate (8-SPT, Sigma), a nonspecific AR antagonist[20]; 100 μmol/L 5′-N-ethylcarboxamidoadenosine (NECA, Sigma), a nonspecific AR agonist[7]; 200 μmol/L PSB1115 (Tocris), a specific A2bAR antagonist[22]; or saline before the addition of platelet agonist.

Tail Bleeding and Time to Occlusion

Bleeding times were assessed using an adaptation of the method we previously described.[23] For details please see supplemental materials.

Gel Permeation Chromatography

5′-NT fractions were separated by gel permeation chromatography on a Nucleogel GFC 1000 to 8 column (Machery-Nagel). For details please see supplemental materials. Each fraction (50 μL) was added to whole human blood diluted 1:2 with saline before the addition of ADP and percent aggregation was measured.

Adenosine Measurements

2 mL of heparinized blood in the presence or absence of 0.2 mmol/L dipyridamole (a nonspecific inhibitor of equilibrative nucleoside transporters; GensiaSicor) was treated with 5U/mL 5′-NT or saline and immediately added to 6 mL of 0.6N perchloric acid at 0°C. Adenosine concentrations were measured in perchloric acid extracts from whole blood as described previously.[24] For details please see supplemental materials.

Statistical Analysis

All values are presented as the mean±SD of n independent experiments. All data were subjected to Student unpaired *t* test or 1-way ANOVA, followed by the Student-Newman-Keuls posthoc test using SigmaStat software (SPSS). Differences were considered significant at $P<0.05$.

Results

Soluble 5′-NT Inhibits Platelet Aggregation in Whole Human Blood

Based on recent studies suggesting soluble 5′-NT purified from *Crotalus atrox* venom for treatment of a broad range of clinical conditions[5–8,10–12] and studies demonstrating the possible role of ecto-5′-nt in thromboregulation,[10,18,25] we examined the effect of 5′-NT on platelet aggregation using a whole blood aggregation model. For this purpose, we used whole blood donated from human volunteers and added soluble 5′-NT or saline. Treatment with soluble 5′-NT resulted in platelet aggregation that was 100% inhibited when aggregation was induced by collagen, ADP (Figure 1A and 1B, respectively), arachidonic acid, or epinephrine and partially inhibited when induced by the potent platelet agonist thrombin (supplemental Figure IA through IC, respectively). These data reveal for the first time that soluble 5′-NT derived from *Crotalus atrox* venom inhibits aggregation in whole human blood.

Biological Activity Within Soluble 5′-NT

To confirm that the antiaggregational effects of commercially available 5′-NT are conferred by a single biologically active

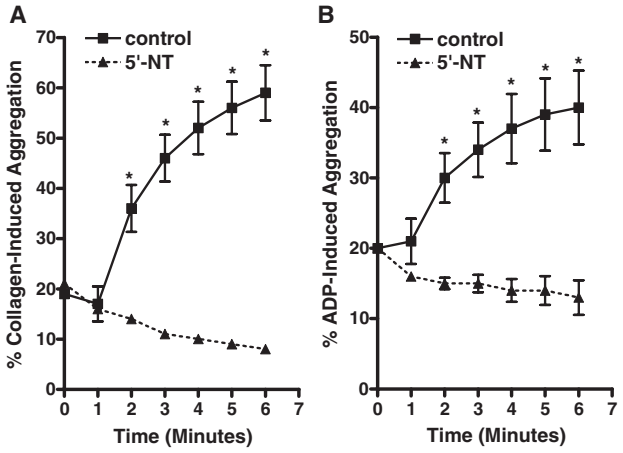

Figure 1. Soluble 5′-NT inhibits human platelet aggregation. 5U/mL 5′-NT or saline (control) was added to human blood. Percent aggregation was measured in response to (A) 5 μg/mL collagen or (B) 10 μmol/L ADP. Each point represents the mean±SD from 4 to 6 experiments (*$P<0.05$ vs 5′-NT treatment).

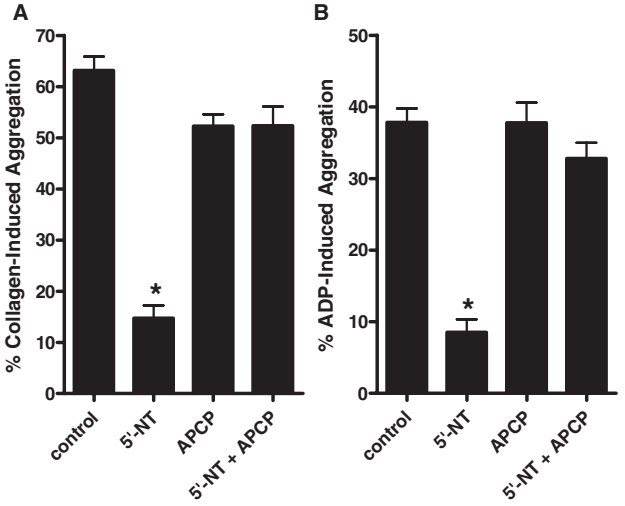

Figure 3. 5U/mL 5′-NT or saline (control) was added to human blood in the absence/presence of 500 μmol/L APCP, a 5′-NT inhibitor. Aggregation (after 6 minutes) was measured in response to (A) 5 μg/mL collagen or (B) 10 μmol/L ADP. Each bar represents the mean±SD from 6 to 8 experiments (*$P<0.05$ vs other groups).

component, we fractionated 5′-NT by gel permeation chromatography, collected 5 fractions, and tested the percent aggregation of each fraction. As shown in Figure 2A, purification yielded 2 major peaks and 3 minor ones. However, only fraction 1 significantly inhibited aggregation similar to unfractionated 5′-NT (Figure 2B). Platelet aggregation curves were similar to those in Figure 1 (data not shown). In contrast, all other fractions had no significant effect on whole-blood aggregation (Figure 2B). These results suggest that the antiaggregational activity of 5′-NT from *Crotalus atrox* venom is appropriated by a single biological activity, which can be isolated by chromatographic techniques.

Ecto-Nucleotidase Inhibitor APCP Blocks Anticoagulatant Effects of 5′-NT

We next investigated aggregation in whole human blood treated with APCP, a specific ecto-5′-nucleotidase inhibitor or saline. As shown in Figure 3, 5′-NT completely inhibited aggregation. Platelet aggregation was not influenced by the addition of APCP alone. However, when APCP was added together with 5′-NT, APCP fully restored collagen- or ADP-induced aggregation (Figure 3A and 3B, respectively) suggesting that the antiaggregational effects of soluble 5′-NT are caused by ecto-nucleotidase effects (as opposed to nonspecific effects).

Adenosine Levels Are Elevated in Whole Blood With 5′-NT Treatment

Because of the fact that extracellular adenosine is rapidly taken up by adenosine transporters, we performed these studies in the presence or absence of dipyridamole, an adenosine uptake inhibitor. As shown in Figure 4, adenosine levels in whole blood were significantly increased after 5′-NT treatment compared to saline-treated blood. As expected, inhibition of adenosine uptake into cells with dipyridamole resulted in significantly higher levels of adenosine when blood was treated with 5′-NT. These studies reveal that treatment of whole blood with soluble 5′-NT results in significant elevation of whole blood adenosine levels.

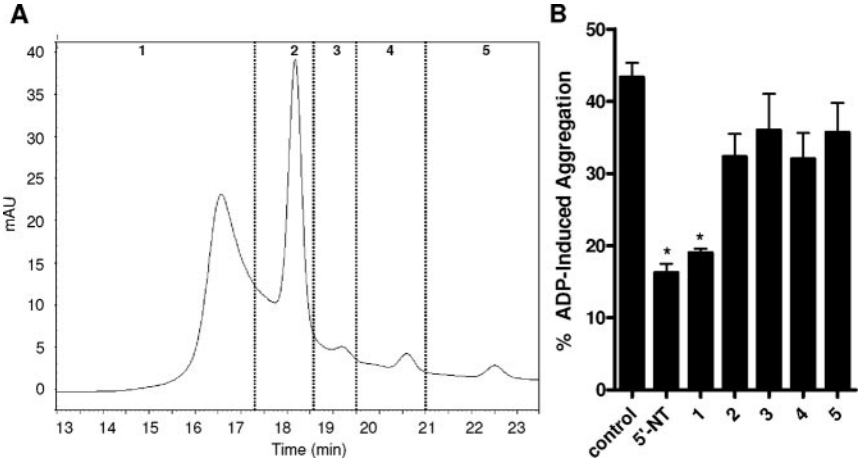

Figure 2. (A) 5′NT was fractionated by gel permeation chromatography, and fractions 1 to 5 were collected. (B) Percent ADP-induced aggregation after 6 minutes in whole human blood after addition of saline (control), 5U/mL 5′NT, or 50μL of each fraction. Each bar represents the mean±SD from 3 to 4 experiments (*$P<0.05$ vs control).

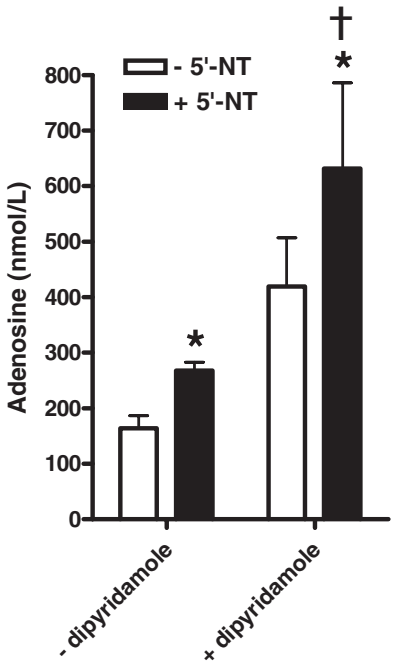

Figure 4. Adenosine levels increase after 5′-NT treatment. Heparinized human blood in the presence/absence (±) of dipyridamole was treated with/without (±) 5U/mL 5′-NT and added to 0.6N perchloric acid. Adenosine was measured in extracts and expressed as the mean±SD from 6 experiments (*$P<0.05$ vs 5′-NT; †$P<0.05$ vs dipyridamole).

Adenosine Receptor Signaling After 5′-NT Treatment

To confirm that extracellular adenosine signaling is responsible for the observed antiaggregational effects of soluble 5′-NT, we pursued aggregational studies using the nonspecific AR antagonist 8-SPT. Platelet aggregation was not influenced by pretreatment with 8-SPT alone (Figure 5A and 5B). However, inhibition of platelet aggregation by 5′-NT in collagen- or ADP-induced aggregation was completely reversed with 8-SPT suggesting that 5′-NT inhibits aggregation via adenosine signaling.

It has been suggested that inhibition of platelet aggregation by adenosine occurs via A2 receptor stimulation.[26,27] To further investigate the role of the A2ARs, we measured aggregation of $A2a^{+/+}$ and $A2a^{-/-}$ mouse blood. The nonspecific AR agonist, NECA, inhibited aggregation in $A2a^{+/+}$ but not $A2a^{-/-}$ mouse blood (Figure 5C) suggesting that adenosine inhibits aggregation through the A2aAR. Furthermore, inhibition of aggregation of $A2a^{+/+}$ mouse blood by NECA was partially reversed in the presence of the specific A2b antagonist PSB1115, suggesting a small contribution by the A2bAR.

Effects of 5′-NT In Vivo

After having shown that soluble 5′-NT inhibits aggregation in whole blood ex vivo, we pursued the effects of 5′-NT in vivo. C57BL/6 mice were treated with 5′-NT or saline. After 30 minutes blood was drawn via cardiac puncture and aggregation was measured. Administration of 5′-NT to mice in vivo abolished collagen- and ADP-induced platelet aggregation ex vivo (Figure 6A and 6B, respectively). Furthermore, adenosine levels were significantly increased after 5′-NT treatment compared to saline-treated mice (Figure 6C). As hemostasis involves both platelet aggregation and blood coagulation, we next assessed the role of 5′-NT in hemostasis by measuring bleeding times in mice after tail-tip amputation. Normal bleeding time in mice treated with vehicle (saline) was 7.3±0.5 minutes. After treatment with 5′-NT, the bleeding time significantly increased to 15±2.7 minutes. However, the effect of 5′NT on bleeding time was blocked by the AR antagonist 8-SPT (6.8±0.7 minutes) suggesting that in addition to inhibiting platelet aggregation ex vivo, 5′-NT can also prevent aggregation in vivo.

Discussion

During atherosclerosis, thrombosis is initiated when the plaque develops fissures or tears and exposes the lipid-rich core to blood in the arterial lumen. Platelet adherence to the exposed subendothelium collagen results in platelet activation and release and local accumulation of platelet agonists such as ADP and thromboxane. This in turn causes further platelet aggregation, arterial vasoconstriction, and subsequent reduction in blood flow. Thus, platelets are the major initiators of arterial thrombosis and offer an attractive target for

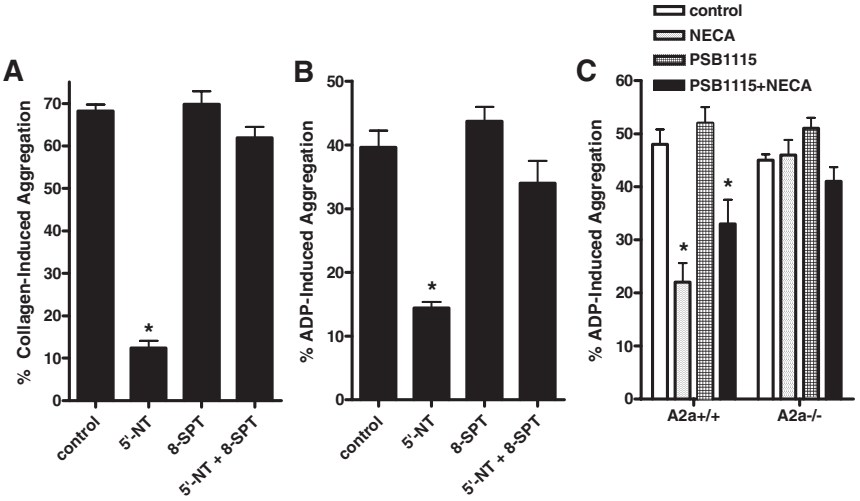

Figure 5. (A) Collagen- or (B) ADP-induced aggregation after control or 5′NT ± 8′-SPT, a nonspecific AR antagonist was added to human blood. *P_0.05 vs other groups. (C) ADP-induced aggregation after NECA (nonspecific AR agonist), PSB1115 (A2b antagonist), or control was added to $A2a^{+/+}$ or $A2a^{-/-}$ mouse blood. *$P<0.05$ vs respective control.

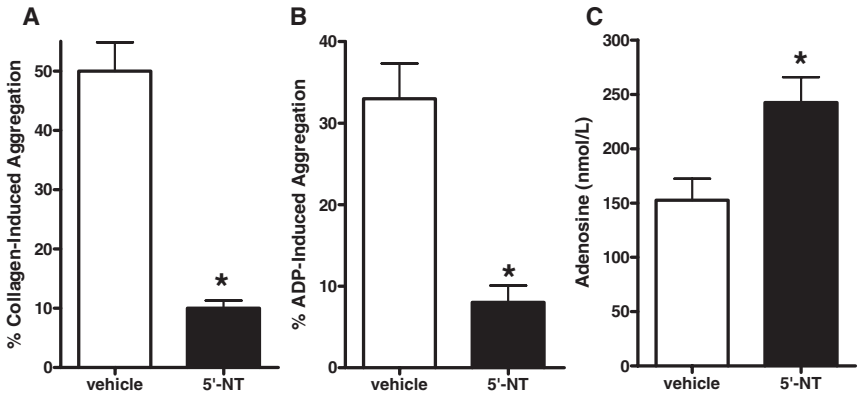

Figure 6. WT mice were treated with saline (vehicle) or 5′-NT (500U/kg, i.p.). After 30 minutes blood was drawn and (A) collagen- or (B) ADP-induced aggregation from mean±SD 6 experiments was measured. *$P<0.05$ vs vehicle. (C) Adenosine concentrations expressed as the mean± SD from 4 to 6 experiments. *$P<0.05$ vs vehicle.

prevention and treatment of thrombosis. Antiplatelet therapy is important for treatment or prophylaxis of conditions like myocardial infarction, stroke, and other vascular diseases.

It has been previously recognized that adenosine is a key mediator in inhibition of platelet aggregation.[14–17] Our results further support this data. Additionally we show using whole blood that soluble 5′-NT inhibits the ability of platelets to aggregate in the presence of other cells via production of adenosine. Furthermore, we demonstrate that treatment with 5′-NT in vivo inhibits aggregation and hemostasis.

This raises the question as to which cells in whole blood are responsible for production of adenosine during 5′-NT treatment. Adenosine is produced by a variety of cells, including leukocytes, smooth muscle cells, and, most notably, endothelial cells (ECs). Thus, B lymphocytes are capable of actively degrading ATP to adenosine,[28] and adenosine can be generated via CD39 and CD73 expressed on CD4+/CD25+ regulatory T cells.[29] However, it is likely that 5′-NT inhibits aggregation via its action on ECs. Activated platelets release ADP which is subsequently metabolized to AMP by CD39 on the EC surface where this cell–cell interaction inhibits further platelet recruitment and aggregation.[15,16,30] The interaction between neutrophils with local ECs may additionally result in adenosine production.[31,32] Alternatively, arterial smooth muscle cells may be responsible for adenosine generation.[33]

Our results demonstrate a significant but modest increase in adenosine levels after 5′-NT treatment. One of the reasons may be because of the short half life of adenosine.[34,35] Furthermore, adenosine cannot only be taken up by ENTs,[36] but also is metabolized to inosine via adenosine deamination.[37] Thus, inosine levels may potentially be elevated by 5′-NT treatment. However, in the local environment where platelets and other cells release ATP (ie, leukocytes, lytic cells), adenosine concentrations can be higher.[31,32,38,39]

It is believed that the antiaggregatory action of adenosine depends on the inhibition, coupled with adenylate cyclase activation, and thus increased cAMP concentrations for both calcium influx and mobilization of internal stores.[40] Platelets represent a uniform tissue possessing A2 receptors (now known to be the A2a receptor subtype) on the external membrane.[41] The inhibition of platelet aggregation by adenosine is thought to be mediated by the stimulation of adenylate cyclase through A2a receptors expressed on platelets.[26] However, it has also been reported that gene expression of the A2b receptor was similar to that of the A2a receptor in human platelets and that the A2b receptor was expressed at the protein level and to be of functional importance.[27] Our results show that 8′-SPT, a nonspecific adenosine receptor antagonist reversed the inhibitory effects of 5-NT, suggesting that 5-NT inhibits aggregation via adenosine binding to adenosine receptors on platelets. Furthermore, we show that a nonspecific AR agonist inhibited aggregation in $A2a^{+/+}$ but not $A2a^{-/-}$ mouse blood and that a specific A2b antagonist partially reversed this inhibitory effect suggesting that 5′-NT may inhibit aggregation via the A2aAR with a small contribution by the A2bAR.

ADP plays a key role in hemostasis because of its ability to stimulate platelet aggregation and, when secreted from platelet-dense granules, potentiates the aggregation response induced by other agents.[42] Thus, any maneuver which deletes ADP will result in inhibition of further activation and recruitment.[15,16,30] ADP-induced platelet activation involves at least 2 receptors.[39] The purinergic $P2Y_1$ receptor, which is coupled to Gαq and mobilization of intracellular calcium, mediates platelet shape change and initiates aggregation, whereas another, the $P2Y_{12}$ receptor, coupled to Gi_2 and adenylyl cyclase inhibition, is responsible for completion and amplification of the response.[43] Therefore it is also possible that 5′-NT may be inhibiting or blocking the $P2Y_1$ receptor (the initiating aggregation receptor) to some extent. Further studies will need to be performed to confirm this.

The current standard of care for the treatment of arterial thrombosis includes anticoagulants and 3 classes of antiplatelet agents including aspirin, thienopyridines, and glycoprotein IIb-IIIa antagonists. Although these drugs have had a significant impact on morbidity and mortality in several patient populations, up to 15% to 25% of high-risk patients with acute coronary syndrome continue to suffer from ischemic events.[44] This problem may occur, in part, because the platelets in many patients are nonresponsive to aspirin and clopidogrel.[44] Additionally, many individuals vary in their platelet response to different drugs and combination antithrombotic therapies have become commonplace. Adenosine has already been recognized as a potential therapeutic agent in treatment of thrombotic disorders.[17,30] Furthermore, many investigators have recently recognized the importance of developing drugs responsible for increasing adenosine and adenosine receptor signaling.[30,45] Thus, 5′-NT may offer an

additional therapeutic approach to increase adenosine levels for the secondary prevention of vascular occlusions.

Murine models have demonstrated that 5′-NT is effective in treatment of vascular leakage secondary to hypoxia[7] and IRI,[5,6,8] and many groups suggest using this enzyme clinically.[5–8,10–12] Until now the important potential side effect of 5′-NT on aggregation has not been considered. This information is crucial when considering 5′-NT as a therapeutic for treatment of ARDS, sepsis, multiple trauma, severe liver failure, severe pancreatitis, lung injury, myocardial infarction, intestinal or renal ischemia, or thrombosis formation. The present study suggests that the effect 5′-NT has on aggregation should be an important factor to consider, especially for those patients who have complications such as excessive postoperative bleeding.

Collectively we demonstrate that treatment of human blood with 5′-NT inhibits aggregation via production of adenosine. Furthermore, we demonstrate that treatment with 5′-NT in vivo inhibits aggregation and hemostasis. These findings have 2 important implications. First, antiaggregational effects of soluble 5′-NT have to be considered as an important side-effect when using 5′-NT in the treatment of vascular leakage, organ ischemia, or acute lung injury. Second, the antiaggregational effects of soluble 5′-NT may serve as an additional therapeutic approach for the treatment of excessive aggregation or thrombosis.

Acknowledgments

The authors gratefully acknowledge Edgar Hoffmann and Renate Riehle for technical assistance.

Sources of Funding

This work was supported by German Research Foundation grants EL274/2-2, EL 274/4-1 to H.K.E.; Foundation for Anesthesia Research and Education grant to H.K.E.; Fortuen grant D3008762 to M.L.H. and H.K.E.; IZKF Tuebingen Nachwuchsgruppe grant 1605-0-0 to T.E.; European Society for Anaesthesiology Research grant D3008762 to M.L.H. and H.K.E.

Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Direct Treatment of Mouse or Human Blood With Soluble 5′-Nucleotidase Inhibits Platelet Aggregation

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Arterioscler Thromb Vasc Biol. 2008;28:1477-1483; originally published online May 29, 2008;
doi: 10.1161/ATVBAHA.108.169219
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

On-line Supplemental Methods

Mice

All animal experiments were conducted under a protocol approved by the Harvard Medical Area standing committee on animals at Brigham and Women's Hospital in accordance with the U.S. National Institutes of Health or in agreement with the German guidelines for use of live animals approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. C57BL/6 mice (aged 8-12 weeks) were treated intraperitoneally (i.p.) with 500 U/kg 5'-NT with or without 40 mg/kg 8-(p-sulfophenyl)theophylline hydrate (8-SPT, Sigma, St. Louis, MO), a non-specific adenosine receptor (AR) antagonist [1] or an equivalent volume of saline. After 30 min, mice were anesthetized with sodium pentobarbital (70 mg/kg) and blood was drawn via cardiac puncture into a 0.1 volume of sodium citrate (3.8%) or tail bleeding time was assessed as described below. In additional experiments, $A2a^{+/+}$ or $A2a^{-/-}$ [2] mouse blood was used to test aggregation *ex vivo.*

Whole Blood Aggregation

Whole blood aggregation studies were completed on the 560VS aggregometer (Chronolog, Havertown, PA) within 3 h of collection. The protocol for blood donations from healthy volunteers was approved by the institutional review board and written informed consent was obtained from each individual donor prior to blood donation. Blood from healthy human volunteers or mice was drawn into a

0.1 volume of sodium citrate (3.8%), subsequently diluted 1:2 with saline and preincubated at 37°C for 5 min in an aggregometer cuvette (Chronolog, Havertown, PA). Platelet activation was started by the addition of one of the following platelet agonists: 5 µg/mL collagen, 10 µM ADP, 50 µM epinephrine, 0.5 µM arachidonic acid, or 0.1 U/mL thrombin (Chronolog, Havertown, PA) and percent aggregation was measured for 6 min. In some experiments, whole blood was treated with 5 U/mL 5'-NT purified from *Crotalus atrox* venom (Sigma) with and without 500 µM 5'-[αβ-methylene] diphosphate (APCP, Sigma), a specific inhibitor of ecto-5'-NT [3]; 200 µM 8-SPT (Sigma), a non-specific AR antagonist [1]; 100 µM 5'-N-ethylcarboxamidoadenosine (NECA, Sigma), a non-specific AR agonist [4]; 200 µM PSB1115 (Tocris, Ellisville, MO), a specific A2bAR antagonist [5];or saline prior to the addition of a platelet agonist.

Tail Bleeding and Time to Occlusion

Bleeding times were assessed using an adaptation of the method we previously described [6]. Briefly, mice were anesthetized with sodium pentobarbital (70 mg/kg body weight, i.p.) and placed on a temperature-controlled heating table to maintain body temperature at 37°C. The mice were then secured with their tails facing downward and perpendicular to their bodies. After being pulled through a 1.5-mm-diameter template, the tails were transected with a scalpel blade and bled onto a Whatman filter paper. The filter paper was dabbed to the wound every 30 seconds without disrupting the forming clot. Any blood dripping during

the 30-sec intervals was allowed to drop freely onto the filter. The experiment was continued until bleeding stopped completely. If bleeding continued after 20 min, bleeding was stopped by cauterization to prevent hypovolemic shock.

Gel Permeation Chromatography

5'-NT (30 U x 2) fractions were separated by gel permeation chromatography on a Nucleogel GFC 1000-8 column (Machery-Nagel, Düren, Germany). The mobile phase consisted of 1 L of water containing 19 mM potassium dihydrogen phosphate and 45 mM sodium chloride (pH 7.0). With a flow rate of 0.6 mL/min and a wavelength of 215 nm, the fractions for each peak were collected, evaporated to dryness by a lyophilizator, and reconstituted in 300 µl of water (pH 7.4). The units for each peak were not determined. Each fraction (50 µl) was added to whole human blood (diluted 1:2 with saline) prior to the addition of ADP and percent aggregation was measured.

Adenosine Measurements

2 mL of heparinized blood in the presence or absence of 0.2 mM dipyridamole (GensiaSicor, Irvine, CA, a non-specific inhibitor of equilibrative nucleoside transporters) was treated with 5 U/mL 5'-NT or saline and immediately added to 6 mL of 0.6 N perchloric acid at 0°C. The tubes were vortexed for 1 minute, placed on ice, and centrifuged within 10 min at 3,350 *g* for 10 min at 4°C. Adenosine concentrations were measured in perchloric acid extracts from whole blood as described previously [7]. Briefly, all samples were supplemented with a

known amount of *N*-6-methyladenosine as the internal standard. The supernatant was adjusted to a pH of 5.5-6.5 by adding 2 M potassium carbonate. The precipitated potassium perchlorate was discarded after centrifugation at 20,000 *g* and the supernatant was applied onto a solid-phase extraction column (BondElut, ICT, Bad Homburg, Germany). Elution of the compounds was performed with 0.1 M HCl, and the eluate was analyzed by HPLC with UV detection using a Nucleosil 100 C18 (3 µm, 125x4 mm i.d.) column. Eluent consisted of *solvent A* (10 mM ammonium dihydrogenphosphate and 0.6 M heptanesulfonic acid sodium salt in 3% methanol) as the ion-pair forming agent and *solvent B* (*solvent A* containing 10% acetonitrile). Remote control, data acquisition, and quantification of peak areas were performed with Peak Simple Software 3.12 by SRI.

Statistical analysis.

All values are presented as the mean ± standard deviation (SD) of *n* independent experiments. All data were subjected to Student's unpaired *t* test or one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test using SigmaStat software (SPSS). Differences were considered significant at $p < 0.05$.

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On-line additional figure:

Figure I

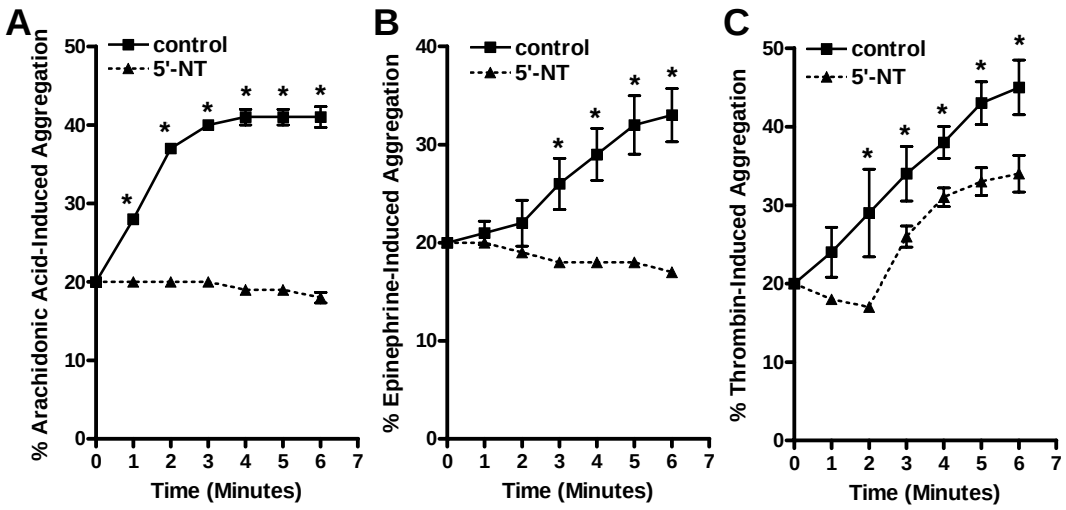

Figure I. Soluble 5'-NT inhibits human platelet aggregation. 5U/mL 5'-NT or saline (control) was added to human blood. Percent aggregation was measured in response to (A) 50µM epinephrine, (B) 0.5µM arachidonic acid, or (C) 0.1U/mL thrombin. Each point represents the mean ± SD from 4-6 different experiments (*, $p<0.05$ vs. 5'-NT treatment).