



HAL
open science

High homocysteine levels prevent via H₂S the CoCl₂-induced alteration of lymphocyte viability

Laurie Bruzzese, Emmanuel Fenouillet, Julien Fromonot, Jm Durand-Gorde, Jocelyne Condo, Nathalie Kipson, Giovana Mottola, Pierre Deharo, Régis Guieu, Jean Ruf

► **To cite this version:**

Laurie Bruzzese, Emmanuel Fenouillet, Julien Fromonot, Jm Durand-Gorde, Jocelyne Condo, et al.. High homocysteine levels prevent via H₂S the CoCl₂-induced alteration of lymphocyte viability. Journal of Cellular and Molecular Medicine, 2016, 10.1111/jcmm.12829 . hal-01307326

HAL Id: hal-01307326

<https://amu.hal.science/hal-01307326>

Submitted on 13 May 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

High homocysteine levels prevent *via* H₂S the CoCl₂-induced alteration of lymphocyte viability

Laurie Bruzzese^{a, #}, Emmanuel Fenouillet^{a, b, #}, Julien Fromonot^{a, c}, Josée-Martine Durand-Gorde^a, Jocelyne Condo^a, Nathalie Kipson^a, Giovanna Mottola^{a, c, d}, Pierre Deharo^{a, d}, Régis Guieu^{a, c}, Jean Ruf^{a, e, *}

^a UMR-MD2, Marseille Medical School, Aix-Marseille University and IRBA, Northern sector, Marseille, France

^b CNRS, Institut des Sciences Biologiques, France

^c Laboratory of Biochemistry, Timone University Hospital, Marseille, France

^d Cardiology Department, Timone University Hospital, Marseille, France

^e INSERM, France

Received: November 25, 2015; Accepted: February 5, 2016

Abstract

High homocysteine (HCy) levels are associated with lymphocyte-mediated inflammatory responses that are sometimes in turn related to hypoxia. Because adenosine is a potent lymphocyte suppressor produced in hypoxic conditions and shares metabolic pathways with HCy, we addressed the influence of high HCy levels on the hypoxia-induced, adenosine-mediated, alteration of lymphocyte viability. We treated mitogen-stimulated human lymphocytes isolated from healthy individuals and the human lymphoma T-cell line CEM with cobalt chloride (CoCl₂) to reproduce hypoxia. We found that CoCl₂-altered cell viability was dose-dependently reversed using HCy. In turn, the HCy effect was inhibited using DL-propargylglycine, a specific inhibitor of the hydrogen sulphide (H₂S)-synthesizing enzyme cystathionine- γ -lyase involved in HCy catabolism. We then addressed the intracellular metabolic pathway of adenosine and HCy, and the role of the adenosine A_{2A} receptor (A_{2A}R). We observed that: (i) hypoxic conditions lowered the intracellular concentration of HCy by increasing adenosine production, which resulted in high A_{2A}R expression and 3', 5'-cyclic adenosine monophosphate production; (ii) increasing intracellular HCy concentration reversed the hypoxia-induced adenosinergic signalling despite high adenosine concentration by promoting both S-adenosylhomocysteine and H₂S production; (iii) DL-propargylglycine that inhibits H₂S production abolished the HCy effect. Together, these data suggest that high HCy levels prevent, *via* H₂S production and the resulting down-regulation of A_{2A}R expression, the hypoxia-induced adenosinergic alteration of lymphocyte viability. We point out the relevance of these mechanisms in the pathophysiology of cardiovascular diseases.

Keywords: adenosine • A_{2A} receptor • CoCl₂ • H₂S • homocysteine • hypoxia • lymphocyte

Introduction

Homocysteine (HCy) is a thiol-containing amino-acid intermediate produced during the synthesis of cysteine from methionine [1]. High HCy concentrations are associated with lymphocyte-mediated inflammatory responses driven by immune mediators [2–4]. HCy metabolism is also linked to the metabolism of adenosine [5, 6], a nucleoside that potentially alters lymphocyte viability *via* activation of its A_{2A} receptor (A_{2A}R) [7]. Adenosine has therefore an anti-inflammatory activity

[8] particularly during hypoxia/ischaemia where it is produced in large amounts at the sites of injury [9].

Hyperhomocysteinaemia results from genetic enzymatic deficiencies and/or nutritional defects that affect HCy metabolism [10]. Under basal conditions, adenosine and HCy result from hydrolysis of S-adenosylhomocysteine (SAH) *via* the SAH hydrolase, and in hyperhomocysteinaemia conditions the hydrolase reaction reverses and SAH accumulates at the expense of adenosine. Subsequently, facilitated diffusion of plasma adenosine into the cell through equilibrative nucleoside transporters [11] increases and the adenosine depletion generated by this situation reduces stimulation of its cell surface receptors [12]. This situation could contribute to pathological

[#]Both authors contributed equally.

*Correspondence to: Dr Jean Ruf.
E-mail: jean.ruf@univ-amu.fr

processes [5] by interfering, for example, with cardioprotective, vasodilatory effects resulting from A_{2A}R activation [13, 14] and several epidemiological studies showed that high concentrations of Hcy are indeed associated with cardiovascular diseases [15–17].

Hydrogen sulphide, an end product of Hcy catabolism *via* the transsulfuration pathway, entered recently the family of gasotransmitters along with NO and CO, because of the effects of H₂S at the cellular and molecular level [18]. This gas was also considered as an autocrine/paracrine T-lymphocyte activator [19], and we recently reported that H₂S reverses the adenosinergic alteration of T-lymphocyte viability *via* repression of the NF- κ B, which down-regulates A_{2A}R expression [20].

Based on this finding and on the literature reported above, we undertook to delineate the relationship between Hcy, adenosine and A_{2A}R expression in hypoxic conditions with particular emphasis on the effects of H₂S produced by high Hcy levels on the hypoxia-induced, adenosine-mediated (hypoxia-adenosinergic thereafter) signalling in lymphocytes.

Materials and methods

Peripheral blood lymphocyte (PBL) preparation

Blood samples were collected from brachial vein of three healthy donors (two males and one female, 25, 38 and 42 years old respectively) after written consent. The study methodologies conformed to the standards set by the Declaration of Helsinki. Peripheral blood lymphocyte were isolated according to manufacturer's instructions using the Vacutainer[®] Cell Preparation Tube density gradient system (Beckton Dickinson, Franklin lakes, NJ, USA). Cells (3×10^6 cells/ml) were then incubated in the Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 10% foetal calf serum and penicillin/streptomycin (100 U/ml, 100 μ g/ml) for 1 hr at 37°C under 5% CO₂ in a 25 cm² flask (10 ml/flask). Adherent monocytes were then discarded and PBL present in culture supernatant were examined using the cell viability assay described below.

Cell viability assay

Peripheral blood lymphocyte and CEM cells, a human lymphoma CD4⁺ T-cell line expressing A_{2A}R [21], were cultured in RPMI 1640 medium as described above. Cell viability was monitored in 24-well plates using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The MTT assay produces a yellowish solution that is converted to dark blue, water-insoluble, MTT formazan by oxidoreductase enzymes of living cells [22]. MTT (0.5 mg in 100 μ l of PBS, pH 7.3) was added to each well containing cells (0.5×10^6 cells/ml) 3 hr prior to the end of the 24-hr incubation period in culture medium containing 50–800 μ M CoCl₂ in the presence of phorbol myristate acetate (PMA, 50 ng/ml) and phytohemagglutinin (PHA, 5 μ g/ml) as previously reported [20]. Using the same procedure, we tested the effect of Hcy by incubating cells in a medium containing PMA + PHA and 50–400 μ M Hcy that were added just prior to incubation with 100 μ M CoCl₂ (*i.e.* the ED₅₀ deduced from the previous experiment). In subsequent tests, and prior to the addition of CoCl₂, cells in the culture

medium containing PMA + PHA were treated using 200 μ M Hcy (*i.e.* the maximal effective dose) and 2.5–10 mM DL-propargylglycine (PPG). All the reagents were titrated individually in terms of activity in preliminary experiments to determine a range of active concentrations that were not cytotoxic. After treatment, cells were pelleted (10,000 $\times g$ for 15 min.) and supernatants were discarded. The insoluble violet formazan crystals contained in the cell pellets were dissolved using 1 ml 100% dimethyl sulfoxide and absorbance (*A*) was measured at 550 nm.

Cell culture experiments

CEM cells (0.5×10^6 cells/ml) were seeded in 75-cm² flasks (50 ml/flask) and stimulated using PMA + PHA as above. The hypoxic injury was achieved by adding 100 μ M CoCl₂ to the culture medium for 24 hr. To test the effect of Hcy, cells were treated by adding 200 μ M Hcy just prior to the addition of CoCl₂. To test the effect of 10 mM PPG on cells treated with Hcy, the reagent was added concomitantly to Hcy. In preliminary experiments, effective doses of CoCl₂, Hcy and PPG were determined using the cell viability assay described below. All the drugs were added rapidly and sequentially as follows: PMA + PHA, Hcy, PPG and, *in fine*, CoCl₂ whose presence triggered the hypoxia reaction. After 24-hr incubation in one of the following conditions (PMA + PHA containing culture medium only, idem + CoCl₂, +CoCl₂ and Hcy, +CoCl₂, Hcy and PPG), living cells were counted using the Trypan Blue dye exclusion method, aliquoted and centrifuged (1000 $\times g$ for 5 min.). Cell pellets were then frozen at –80°C until use. All reagents were from Sigma-Aldrich (St. Louis, MO, USA).

A_{2A}R expression

A_{2A}R expression was assessed using a semi-quantitative Western blot procedure described previously [23]. Briefly, cell pellets were lysed using a 4% sodium dodecyl sulphate aqueous solution supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and a 15 min.-sonication treatment at 47 kHz. Cell lysates (0.5×10^6) were diluted in loading buffer (65.2 mM Tris-HCl buffer, pH 8.3, containing 10% glycerol, 0.01% bromophenol blue and 5% 2-mercaptoethanol). Samples were then analysed using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblotting onto polyvinylidene difluoride membrane. Blots were incubated for 20 min. with an anti-A_{2A}R mouse mAb (Adonis; 1 μ g/ml) [24]; an anti- β actin mAb (clone AC-15, Sigma-Aldrich; 0.5 μ g/ml) was used as total protein loading control. Blots were then processed using horseradish peroxidase-labelled antimouse antibodies and chemiluminescent substrate (SuperSignal West Femto; Pierce Biotechnology, Rockford, IL, USA). Densitometry analysis of the A_{2A}R band at 45 kDa was performed with a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY, USA) and the ImageJ software (NIH). Results were expressed as arbitrary units (AU) defined as pixels of one peak versus the sum of peaks (percent).

Adenosine and inosine assays

Intracellular adenosine and inosine concentrations were measured as previously described [25] using a high performance liquid chromatography system equipped with a diode array detector (Chromsystems, Munich, Germany). To prevent adenosine degradation, pellets of frozen

cells (3×10^6) were mixed with 500 μ l of a cold stop solution [0.2 mM dipyrindamole, 4.2 mM ethylenediaminetetraacetic acid, 5 mM erythro-9-(2-hydroxy-3-nonyl) adenine, 79 mM α - β methylene adenosine 5'diphosphates and 1 IU/mL heparin sulphate in NaCl 0.9%]. Proteins were precipitated using 1 N perchloric acid and the supernatant was injected into a LiChrospher C18 column (Merck, Darmstadt, Germany). Adenosine and inosine were identified in the same run by their elution time and spectrum. Measurements were made by comparison of peak areas versus those obtained using adenosine and inosine standard solutions. The intra-assay and interassay coefficients of variation ranged from 1% to 3% for both products.

Adenosine deaminase (ADA) activity

Adenosine deaminase activity was measured as previously described [23]. Briefly, cells (1.5×10^6) were lysed using 250 μ l of ultra-pure water, mixed with 750 μ l of 28 mM adenosine in 0.9% NaCl and incubated for 36 min. at 37°C. The reaction was stopped by immersion of the samples in ice water. Ammonium production resulting from adenosine degradation by ADA was determined using a Synchron LX 20 analyser (Beckman Coulter Inc., Villepinte, France). The intra-assay and interassay coefficients of variation ranged from 3% to 5%.

HCy and SAH assays

HCy and SAH concentrations in cell pellets were measured using dedicated ELISA kits (Cusabio, Wuhan Huamei Biotech Co., Ltd, Wuhan, Hubei Province, China). These assays use a quantitative sandwich enzyme immunoassay technique where HCy or SAH are trapped onto an antibody-coated plate prior to detection using a biotin-conjugated antibody specific for HCy or SAH and an avidin-conjugated horseradish peroxidase. For these assays, 2.5×10^6 and 0.5×10^6 cells for HCy and SAH, respectively, were lysed using 100 μ l of 0.25% dodecyl trimethyl ammonium bromide in the assay buffer prior to transfer to microplate according to manufacturer's instructions.

H₂S assay

H₂S production by cells was measured using the methylene blue method following trapping of the sulphides present in the culture medium using zinc acetate in alkaline conditions [26]. Briefly, culture supernatants (50 ml; 0.5×10^6 cells/ml) were mixed with 0.5 g of Zn acetate and 630 μ l NaOH 10 N prior to centrifugation at $3000 \times g$ for 10 min. The supernatants were removed by decantation and the Zn sulphide pellets were washed once with 25 ml ultra-pure water. The pellets were then suspended in 1 ml ultra-pure water and vigorously shaken. One hundred microlitres of 20 mM N,N dimethyl-*p*-phenylenediamine sulphate in 7.2 N HCl was then added followed by 100 μ l of 30 mM FeCl₃ in 1.2 N HCl and the tubes were shaken again. After 20 min. incubation at room temperature in the dark, the samples were centrifuged and the resulting methylene blue dye in the supernatant was measured at 670 nm. The calibration curve of absorbance versus sulphide concentration was obtained using known concentrations of NaHS, a H₂S donor. The H₂S concentration was taken as 30% of the NaHS concentration in the calculation [27].

3', 5'-cyclic AMP (cAMP) assay

The concentrations of cAMP present in cell pellets were measured using the Amersham Biotrak kit (GE Healthcare Life Sciences, Buckinghamshire, UK) that combines the use of a peroxidase-labelled cAMP conjugate and a specific antiserum immobilized on microplates. Cells (1×10^6) were lysed using 100 μ l of 0.25% dodecyl trimethyl ammonium bromide in the assay buffer prior to transfer to microplate wells to carry out the competitive enzyme immunoassay according to manufacturer's instructions.

Statistical analysis

Each assay performed in duplicate was repeated three times. Data (mean \pm S.D.) were compared using the one-way ANOVA with the Fisher's least significant difference test. Differences with $P < 0.05$ were considered to be statistically significant.

Results

HCy prevents the hypoxia-induced alteration of lymphocyte viability

Freshly isolated PBL were used to address the authentic cell situation, whereas the human lymphoma T-cell line CEM was used because it constitutes a cell material whose characteristics are well documented and stable. We mimicked classically the inflammatory context using PMA (5 μ g/ml) and PHA (50 ng/ml), and we reproduced the hypoxia-induced effects using a 50–800 μ M CoCl₂ treatment, a widely used method preventing HIF-1 α degradation [28,29] that was recently validated in much detail in this exact cellular model [20]. CoCl₂ also mimics hypoxia/ischaemia responses by inducing oxidative stress and inflammation [30–33]. Various parameters (cell density, CoCl₂ concentration, treatment duration) were tested in preliminary experiments (data not shown) using the MTT assay to determine the conditions used subsequently. We then choose a low cell density (0.5×10^6 cells/ml) to avoid cell confluency during the experiment, and a long time exposure (24 hr) to drugs (CoCl₂, HCy, PPG) to mimic a chronic situation. As previously obtained with more cells and a shorter time [20]. Figure 1 shows that CoCl₂ treatment affected in a dose-dependent manner the viability of resting PBL (Fig. 1A) and CEM cells (Fig. 1B), PMA + PHA stimulation further increasing the CoCl₂ effect. We next tested the effect of 50–400 μ M HCy on lymphocyte viability in a PMA + PHA-containing culture medium in the presence, or in the absence, of CoCl₂. A 100 μ M CoCl₂ concentration was chosen to mimic hypoxic conditions because it corresponded to the concentration that reproducibly induced the half-maximal effect on stimulated PBL (Fig. 1A) and CEM cell (Fig. 1B) viability. In the absence of CoCl₂, HCy did not affect cell viability, whereas the effect resulting from CoCl₂ treatment for 24 hr was reversed in a dose-dependent manner using HCy in PBL (Fig. 1C)

and CEM cell cultures (Fig. 1D). We previously obtained similar results using NaHS, a H₂S donor [20].

These data indicate that high HCy levels promoted lymphocyte survival in hypoxic conditions, and we used subsequently the maximal effective concentration (200 μM) of HCy.

PPG modulation of HCy effect

PPG is a specific inhibitor of cystathionine-γ-lyase (CSE), and consequently an agent that represses the CSE-mediated H₂S production [34]. To address the mode of action of HCy, we examined using 2.5–10 mM PPG whether HCy acted *via* its degradation product H₂S that down-regulates A_{2A}R expression [20]. Using a high concentration (200 μM) of HCy, and in a context mimicking chronic hypoxia (24 hr incubation with 100 μM CoCl₂), PPG treatment decreased in a dose-dependent manner the viability of stimulated PBL (Fig. 2A) and CEM cells (Fig. 2B) versus the ‘hypoxia + hyperhomocysteinemia’ condition.

These data suggest that the effect of HCy on cell viability was reversed by CSE inhibition, and resulted therefore from H₂S produc-

tion. To further delineate this mechanism, we used in the next experiments the highest concentration of PPG (10 mM) we tested.

Adenosine metabolism

In hypoxic conditions, adenosine is produced intracellularly from ATP and can be secreted *via* equilibrative nucleoside transporters [35]. In the cell, adenosine can be degraded into inosine by ADA. Adenosine can be also produced in the extracellular environment by the ectoATPase/apyrase CD39 and ectonucleotidase CD73 [36] prior to its degradation by ADA associated at the cell surface *via* CD26 [37]. In lymphocyte population, extracellular adenosine can be produced from ATP by the CD4⁺, CD25⁺ regulatory T cells that express both CD39 and CD73 at high levels [38].

Here, we focused on the intracellular adenosine metabolism by testing adenosine and inosine levels as well as ADA activity in lysates of stimulated CEM cells cultured in various conditions. The results are given in Figure 3A: (i) CoCl₂ treatment induced a rise in cellular concentrations of adenosine and inosine (4.17 *versus* 2.68 μM and 9.09 *versus* 2.67 μM in basal conditions respectively), the latter resulting

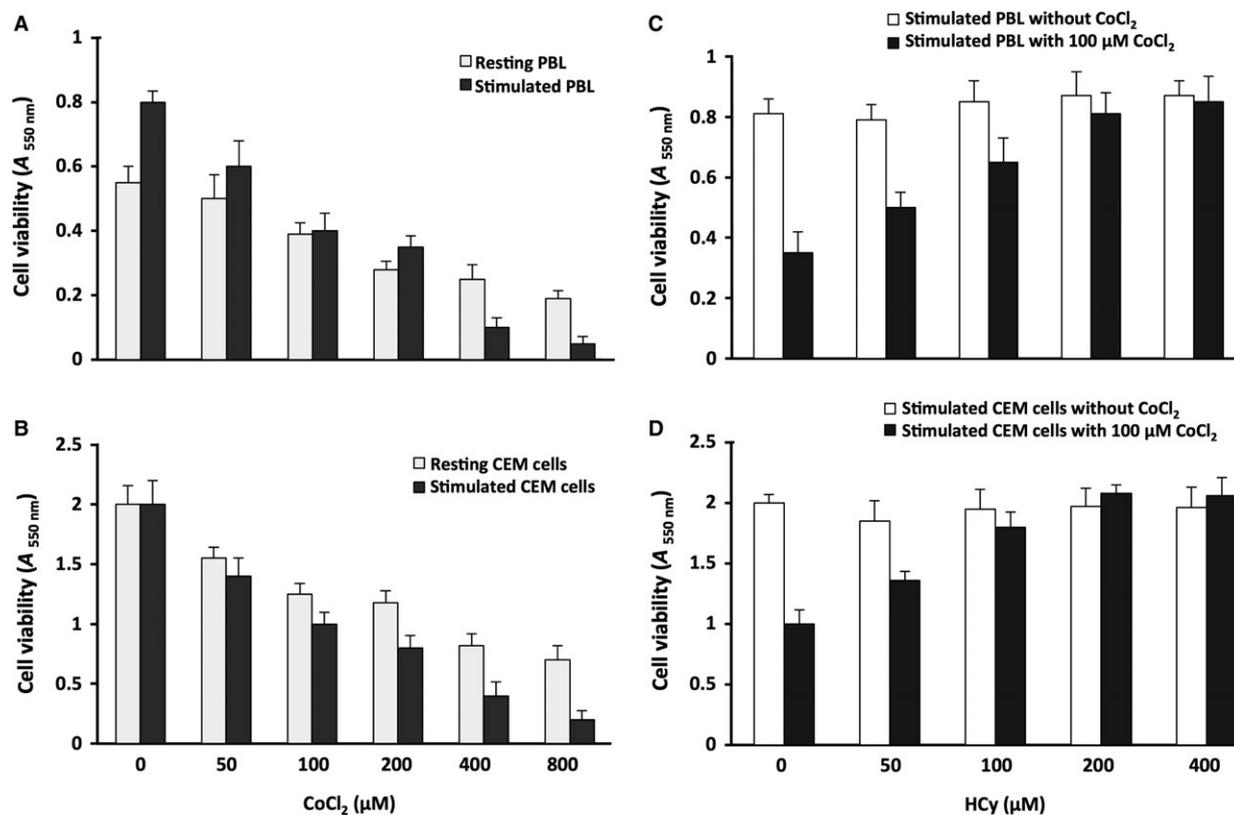


Fig. 1 Cell viability following CoCl₂- and HCy-treatments. The viability index of PBL (A) and CEM cells (B), resting or stimulated using PMA+PHA and incubated with 50–800 μM CoCl₂ for 24 hr was monitored using the MTT assay. Incubation with 50–400 μM HCy in the presence, or not, of 100 μM CoCl₂ was similarly tested using stimulated PBL (C) and CEM cells (B). The dark blue dye produced by living cells was spectrophotometrically measured. Data are given as A_{550 nm} value (mean ± S.D., n = 3).

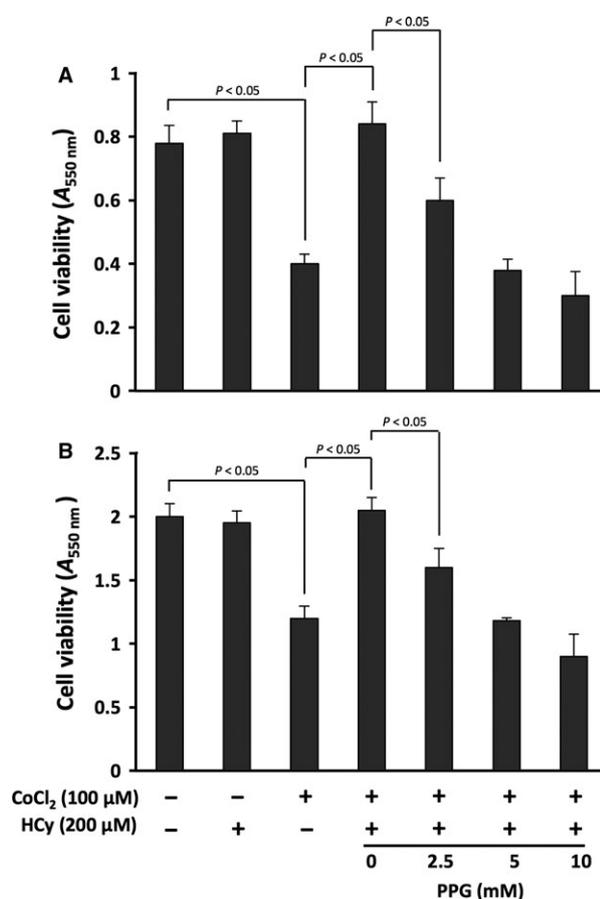


Fig. 2 Effect of PPG in hypoxia and hyperhomocysteinemia conditions. PMA+PHA-stimulated PBL (A) and CEM cells (B) treated with 100 μM CoCl₂ and 200 μM Hcy for 24 hr were incubated with 2.5–10 mM PPG. The viability index was monitored using the MTT assay. Data are given as A_{550 nm} value (mean ± S.D., n = 3). Controls were cells incubated with/without 100 μM CoCl₂ and/or 200 μM Hcy. Statistical significance (P < 0.05) was indicated by brackets where applicable.

probably from the increase in ADA activity (6.68 versus 3.03 IU) produced to counteract the elevated adenosine production generated by hypoxia; (ii) a treatment combining CoCl₂ and Hcy tended to further increase the concentration of adenosine generated by hypoxia (5.50 versus 4.17 μM in hypoxia) while ADA activity and the resulting inosine concentration decreased (2.99 versus 6.68 IU and 8.23 versus 9.09 μM respectively); (iii) a treatment combining CoCl₂ and Hcy together with PPG to inhibit H₂S produced via Hcy catabolism did not modify adenosine concentration (5.32 versus 5.50 μM in the ‘hypoxia + hyperhomocysteinemia’ condition) but increased inosine production (13.10 versus 8.23 μM), a result which was consistent with the parallel increase in ADA activity (6.32 versus 2.99 IU).

These results suggested that the recovery of lymphocyte viability resulting from Hcy treatment (Fig. 1C and D) was not because of a decreased production of adenosine since in fact its intracellular concentration increased (Fig. 3A).

HCy metabolism

During the conversion of methionine to Hcy, S-adenosylmethionine is produced from ATP. After demethylation, SAH is generated and subsequently degraded by the SAH hydrolase into Hcy and adenosine that are quickly used in basal conditions, the enzyme being also able to function in the opposite direction [39]. Hcy may be then remethylated to methionine or it may be condensed with serine to form cystathionine and then cysteine via the transsulfuration pathway [40]. H₂S, an end product of the pathway, is produced by cystathionine β-synthase and CSE from Hcy during T-cell activation [19]. In the following experiments, we focused on H₂S production by CSE because: (i) CSE controls the metabolism of cysteine into H₂S, pyruvate and NH₄⁺ [41]; (ii) CSE is the main player in hyperhomocysteinemia conditions [42] and (iii) CSE can be specifically inhibited using PPG [34].

The Hcy metabolism in lymphocyte was studied by testing Hcy and SAH concentrations in lysates of stimulated CEM cells cultured in various conditions while H₂S intracellularly produced and subsequently secreted in the culture medium was measured in the culture supernatant. Our findings are given in Figure 3B: (i) following CoCl₂ treatment, Hcy and hence H₂S concentration, decreased versus basal conditions (3.01 versus 3.59 μM and 0.81 versus 1.01 μM respectively). Conversely, SAH production increased (4.47 versus 4.07 μM) as a result of the hypoxia-induced production of adenosine; (ii) a treatment combining 100 μM CoCl₂ and 200 μM Hcy in the culture medium markedly increased intracellular Hcy concentration (4.63 versus 3.01 μM in hypoxia); this situation stimulated intracellular SAH production (5.34 versus 4.47 μM), which in turn promoted Hcy degradation via the transsulfuration pathway into H₂S that strongly increased in culture medium (1.31 versus 0.81 μM); (iii) a treatment combining CoCl₂, Hcy and PPG did not significantly alter the concentration of SAH and Hcy (5.03 versus 5.34 μM and 4.61 versus 4.63 μM in the ‘hypoxia + hyperhomocysteinemia’ condition respectively) but strongly decreased H₂S production (0.71 versus 1.31 μM).

These data further support the conclusion that hyperhomocysteinemia reversed the hypoxia-adenosinergic alteration of lymphocyte viability via H₂S production.

Adenosinergic signalling

Adenosine inhibits lymphocyte viability mainly via A_{2A}R signalling and stimulation of adenylyl-cyclase and G_s-proteins [43,44], which controls in turn a plethora of biological processes [45]. We addressed here the hypoxia-adenosinergic lymphocyte signalling by testing A_{2A}R expression, cAMP production and viability of stimulated CEM cells cultured in various conditions. The data are given in Figure 3C: (i) CoCl₂ treatment increased A_{2A}R expression and cAMP production compared with the basal situation (24.0 versus 19.1 AU and 1902 versus 643 fmol/well respectively). This result reflected the binding of adenosine produced by the cell to an increasing number of A_{2A}R,

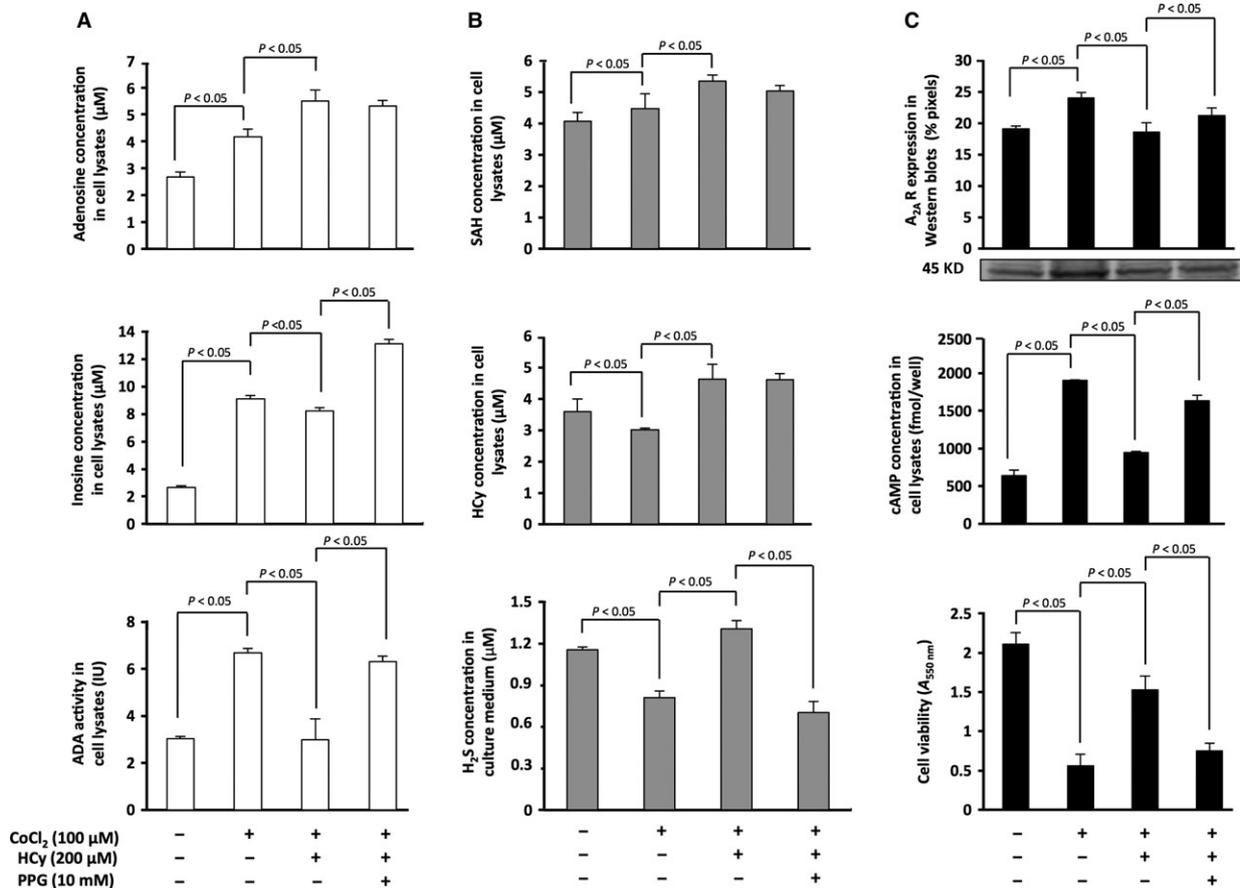


Fig. 3 Metabolism of adenosine and Hcy; adenosinergic signalling. Intracellular adenosine/inosine concentrations, as well as ADA activity, were measured in cell lysates (A). Intracellular SAH and Hcy concentrations were measured in cell lysates, H₂S being measured in culture supernatants (B). A_{2A}R expression, cAMP concentration and viability of the cells were also measured (C). PMA+PHA-stimulated CEM cells were cultured for 24 hr in four experimental conditions: the 'control' condition (stimulated cells), 'hypoxia' (+100 μM CoCl₂), 'hypoxia+hyperhomocysteinaemia' (+200 μM Hcy) and 'hypoxia + hyperhomocysteinaemia' in the presence of 10 mM PPG. Results are mean ± S.D., *n* = 3. Statistical significance (*P* < 0.05) was indicated by brackets where applicable. Specific A_{2A}R bands (45 kDa) from a representative Western blot are shown in the insert.

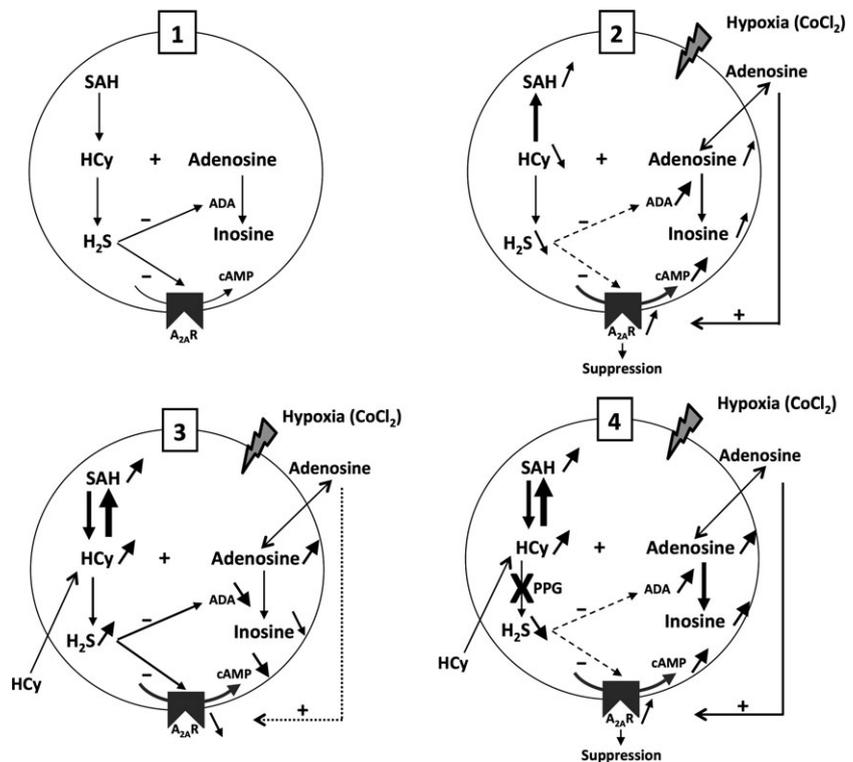
which decreased lymphocyte viability as shown using the MTT assay ($A_{550\text{ nm}} = 0.56$ versus 2.11 in the basal conditions); (ii) a treatment combining CoCl₂ and Hcy reversed the inhibitory effect on viability ($A_{550\text{ nm}} = 1.53$ versus 0.56 in hypoxia) by decreasing A_{2A}R expression and the resulting cAMP production (18.6 versus 24.0 AU and 947 versus 1902 fmol/well respectively); (iii) a treatment combining CoCl₂, Hcy and PPG prevented the effect of Hcy and increased A_{2A}R expression as well as cAMP production (21.2 versus 18.6 AU and 1638 versus 947 fmol/well in the 'hypoxia + hyperhomocysteinaemia' condition respectively), cell viability decreasing ($A_{550\text{ nm}} = 0.75$ versus 1.53) to a value close to that found in the 'hypoxia' condition ($A_{550\text{ nm}} = 0.56$).

These results indicate that the loss of the hypoxia-adenosinergic alteration of lymphocyte viability observed in the presence of Hcy (Fig. 1C and D) mainly depended on the decreased level of A_{2A}R expression (Fig. 3C) resulting from H₂S production (Fig. 3B).

Discussion

We found here that in CoCl₂-induced hypoxic conditions, high Hcy levels increased H₂S production, which down-regulated A_{2A}R expression and repressed the potent hypoxia-adenosinergic alteration of lymphocyte viability. We performed preliminary experiments in which the monitoring of cell viability was used to dissect Hcy regulation of hypoxia-adenosinergic signalling in lymphocytes. We conducted four experimental conditions in culture medium supplemented with PMA + PHA: the control situation, hypoxia, hypoxia and hyperhomocysteinaemia, and hypoxia and hyperhomocysteinaemia in the presence of PPG. We previously reported using the same CEM cell line that hypoxia stimulates adenosinergic signalling [23] and that A_{2A}R stimulation decreases lymphocyte viability [20, 21]. Here, we confirmed these previous data and detailed the metabolic pathways of Hcy and adenosine that are interdependent and involved in the control of lymphocyte viability (see Fig. 4 for an overview).

Fig. 4 Proposed overview of H₂S modulation of the adenosinergic signalling in lymphocytes. Four experimental cell culture conditions are considered: the 'control' condition (PMA+PHA-stimulated cells) (1), 'hypoxia' (2), 'hypoxia+hyperhomocysteinaemia' (3) and 'hypoxia+hyperhomocysteinaemia' in the presence of PPG (4). Up/down arrows represent the increase/decrease in a parameter respectively. The thickness of the arrow reflects the level of the parameter. CoCl₂-mediated hypoxia induces intracellular production of adenosine that can exit the cell through equilibrative nucleoside transporters. Adenosine stimulates A_{2A}R that triggers the cAMP-mediated suppressive signal. H₂S is produced from H₂Cy by CSE that is inhibited by PPG. H₂S represses ADA production and A_{2A}R expression.



Firstly, we found that hypoxia increased intracellular adenosine concentration to form SAH at the expense of H₂Cy *via* the SAH hydrolase. In basal conditions, the hydrolase tends to produce adenosine and H₂Cy from SAH because these products are rapidly catabolized [39]. Under hypoxic conditions, intracellular adenosine synthesis increases, which results in a shift of the enzymatic reaction towards SAH production from adenosine and H₂Cy as shown here. A major consequence is that H₂Cy is no longer catabolized to form H₂S, and this situation prevents the known down-regulation of adenosinergic signalling by H₂S [20]. Taking into account that lymphocytes, in particular T lymphocytes, play a pivotal role in interaction with other immune cells such as monocytes and neutrophils in an inflammatory context, repressing the lymphocyte response in hypoxic condition can contribute to the protective effect of A_{2A}R activation, notably in cardiovascular disease [46]. In the heart, it is also noteworthy that production of adenosine following hypoxia/ischaemia has positive effects on the vasculature *via* the A_{2A}R-mediated coronary vasodilation process [47–49]. Thus, during hyperhomocysteinaemia in hypoxic/ischaemic conditions, the catabolism of H₂Cy into H₂S and its beneficial action on lymphocyte viability may be of particular importance regarding the onset and progression of cardiovascular disorders that generally include a strong inflammatory background [15].

Secondly, when high extracellular concentrations of H₂Cy were added to the cell environment to mimic hyperhomocysteinaemia, H₂Cy entered the cell by transporters similar to those for cysteine and cystine [50]. In these conditions, intracellular H₂Cy was no longer the limiting substrate for the production, together with adenosine, of SAH that strongly increased. The resulting high SAH

concentration then forced the SAH hydrolase-mediated reaction to reverse and produce, as in basal conditions, H₂Cy and adenosine that accumulated into the cell. Excess of intracellular H₂Cy produced from SAH could be in turn degraded to generate H₂S that repressed both ADA activity and A_{2A}R expression. The resulting low ADA activity enabled to increase intracellular adenosine concentration and to decrease inosine production, whereas the low A_{2A}R expression restored the lymphocyte viability.

Thirdly, we further showed that H₂Cy reversed the suppressive effect of hypoxia on lymphocyte viability *via* its catabolism into H₂S, PPG preventing the H₂S inhibitory effect on both ADA activity and A_{2A}R expression. In these conditions, adenosine could then exit the cell to activate the highly expressed A_{2A}R that, in turn, produced high level of cAMP as in hypoxic conditions, whereas excess of intracellular adenosine was catabolized by ADA into inosine. We observed, however, that PPG did not increase intracellular homocysteine level, suggesting that homocysteine was metabolized further into cystathionine by cystathionine β-synthase but not into the H₂S end product that requires the CSE activity. Moreover, the increase in H₂S production triggered by H₂Cy was completely abolished by PPG. These data are significant as they provide additional evidence that CSE is a central player in H₂S production in lymphocytes [41]. By showing using PPG the H₂S-mediated hyperhomocysteinaemia effect on the hypoxia-adenosinergic signalling in lymphocytes, we also support here the pro-inflammatory role attributed to H₂S [51] as well as its repressive action on A_{2A}R expression [20].

These results show that H₂Cy down-regulates A_{2A}R expression in hypoxic conditions *via* H₂S production. We propose that this mecha-

nism has strong implications for immunosuppression, and particularly for patients with cardiovascular diseases and severe hyperhomocysteinaemia that was shown to be associated with inflammation and cardiovascular complications. Our findings may provide clues to explain why hyperhomocysteinaemia constitutes a risk factor in cardiovascular diseases [15–17] and may therefore help to define new therapeutic strategies.

Acknowledgements

We thank Michelle Blas for her expertise in the adenosine/inosine assay. This work was supported by grants from AMU and APHM, France.

References

1. Steed MM, Tyagi SC. Mechanisms of cardiovascular remodeling in hyperhomocysteinemia. *Antioxid Redox Signal*. 2011; 15: 1927–43.
2. Holven KB, Aukrust P, Retterstol K, et al. Increased levels of C-reactive protein and interleukin-6 in hyperhomocysteinemic subjects. *Scand J Clin Lab Invest*. 2006; 66: 45–54.
3. Su SJ, Huang LW, Pai LS, et al. Homocysteine at pathophysiologic concentrations activates human monocyte and induces cytokine expression and inhibits macrophage migration inhibitory factor expression. *Nutrition*. 2005; 21: 994–1002.
4. Gori AM, Corsi AM, Fedi S, et al. A proinflammatory state is associated with hyperhomocysteinemia in the elderly. *Am J Clin Nutr*. 2005; 82: 335–41.
5. Riksen NP, Rongen GA, Blom HJ, et al. Potential role for adenosine in the pathogenesis of the vascular complications of hyperhomocysteinemia. *Cardiovasc Res*. 2003; 59: 271–6.
6. Chen YF, Li PL, Zou AP. Effect of hyperhomocysteinemia on plasma or tissue adenosine levels and renal function. *Circulation*. 2002; 106: 1275–81.
7. Sitkovsky MV, Ohta A. The ‘danger’ sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol*. 2005; 26: 299–304.
8. McPherson JA, Barringhaus KG, Bishop GG, et al. Adenosine A(2A) receptor stimulation reduces inflammation and neointimal growth in a murine carotid ligation model. *Arterioscler Thromb Vasc Biol*. 2001; 21: 791–6.
9. Ely SW, Berne RM. Protective effects of adenosine in myocardial ischemia. *Circulation*. 1992; 85: 893–904.
10. Schroecksnadel K, Frick B, Wirleitner B, et al. Moderate hyperhomocysteinemia and immune activation. *Curr Pharm Biotechnol*. 2004; 5: 107–18.
11. Miura T, Ogawa T, Iwamoto T, et al. Dipyridamole potentiates the myocardial infarct size-limiting effect of ischemic preconditioning. *Circulation*. 1992; 86: 979–85.
12. Zanin RF, Bergamin LS, Braganhol E, et al. Homocysteine modifies extracellular ATP availability in macrophages. *Toxicol In Vitro*. 2013; 27: 2273–8.
13. Shryock JC, Belardinelli L. Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol*. 1997; 79: 2–10.
14. Riksen NP, Rongen GA, Boers GH, et al. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol*. 2005; 25: 109–14.
15. Eikelboom JW, Lonn E, Genest J Jr, et al. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. *Ann Intern Med*. 1999; 131: 363–75.
16. Catena C, Colussi G, Nait F, et al. Elevated homocysteine levels are associated with the metabolic syndrome and cardiovascular events in hypertensive patients. *Am J Hypertens*. 2015; 28: 943–50.
17. Catena C, Colussi G, Url-Michitsch M, et al. Subclinical carotid artery disease and plasma homocysteine levels in patients with hypertension. *J Am Soc Hypertens*. 2015; 9: 167–75.
18. Wang R. Two's company, three's a crowd: Can H₂S be the third endogenous gaseous transmitter? *FASEB J*. 2002; 16: 1792–8.
19. Miller TW, Wang EA, Gould S, et al. Hydrogen sulfide is an endogenous potentiator of

Author contributions

L.B. and E.F. performed the experiments, analysed the data and wrote the paper. J.M.D.G., J.C. and N.K. performed the experiments and analysed the data. J.F., G.M. and P.D. and R.G. analysed the data and wrote the paper. J.R. designed the study, planned the experiments, analysed the data and wrote the paper.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

20. Bruzzese L, Fromonot J, By Y, et al. NF-κB enhances hypoxia-driven T-cell immunosuppression via upregulation of adenosine A (2A) receptors. *Cell Signal*. 2014; 26: 1060–7.
21. By Y, Durand-Gorde JM, Condo J, et al. Monoclonal antibody-assisted stimulation of adenosine A2A receptors induces simultaneous downregulation of CXCR4 and CCR5 on CD4 + T-cells. *Hum Immunol*. 2010; 71: 1073–6.
22. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev*. 2005; 11: 127–52.
23. By Y, Jacquin L, Franceschi F, et al. Fall in oxygen tension of culture medium stimulates the adenosinergic signalling of a human T cell line. *Purinergic Signal*. 2012; 8: 661–7.
24. By Y, Durand-Gorde JM, Condo J, et al. Production of an agonist-like monoclonal antibody to the human A2A receptor of adenosine for clinical use. *Mol Immunol*. 2009; 46: 400–5.
25. Guieu R, Sampieri F, Bechis G, et al. Development of an HPLC/diode array detector method for the determination of human plasma adenosine concentrations. *J Liq Chromatogr*. 1999; 22: 1829–41.
26. Ubuka T. Assay methods and biological roles of labile sulfur in animal tissues. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002; 781: 227–49.
27. Qu K, Chen CP, Halliwell B, et al. Hydrogen sulfide is a mediator of cerebral ischemic damage. *Stroke*. 2006; 37: 889–93.
28. Kanaya K, Kamitani T. pVHL-independent ubiquitination of HIF1alpha and its stabiliza-

- tion by cobalt ion. *Biochem Biophys Res Commun.* 2003; 306: 750–5.
29. **Yuan Y, Hilliard G, Fergusson T, et al.** Cobalt inhibits the interaction between hypoxia-inducible factor- α and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor- α . *J Biol Chem.* 2003; 278: 15911–6.
 30. **Zou W, Yan M, Xu W, et al.** Cobalt chloride induces PC12 cells apoptosis through reactive oxygen species and accompanied by AP-1 activation. *J Neurosci Res.* 2001; 64: 646–53.
 31. **Jung JY, Mo HC, Yang KH, et al.** Inhibition by epigallocatechin gallate of CoCl₂-induced apoptosis in rat PC12 cells. *Life Sci.* 2007; 80: 1355–63.
 32. **Kim KS, Rajogopal V, Gonsalves C, et al.** A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. *J Immunol.* 2006; 177: 7211–24.
 33. **Yang Z, Yang C, Xiao L, et al.** Novel insights into the role of HSP90 in cytoprotection of H₂S against chemical hypoxia-induced injury in H9c2 cardiac myocytes. *Int J Mol Med.* 2011; 28: 397–403.
 34. **Asimakopoulou A, Panopoulos P, Chasapis CT, et al.** Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). *Br J Pharmacol.* 2013; 169: 922–32.
 35. **Parkinson FE, Xiong W.** Stimulus- and cell-type-specific release of purines in cultured rat forebrain astrocytes and neurons. *J Neurochem.* 2004; 88: 1305–12.
 36. **Eltzschig HK, Sitkovsky MV, Robson SC.** Purinergic signaling during inflammation. *N Engl J Med.* 2012; 367: 2322–33.
 37. **Eltzschig HK, Faigle M, Knapp S, et al.** Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. *Blood.* 2006; 108: 1602–10.
 38. **Ohta A, Sitkovsky M.** Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol.* 2014; 5: 304.
 39. **Ueland PM.** Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. *Pharmacy Rev.* 1982; 34: 223–53.
 40. **Bearden SE, Beard RS Jr, Pfau JC.** Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress. *Am J Physiol Heart Circ Physiol.* 2010; 299: 1568–76.
 41. **Vuillefroy de Silly R, Couton F, Poirier N, et al.** Transplant tolerance is associated with reduced expression of cystathionine- γ -lyase that controls IL-12 production by dendritic cells and TH-1 immune responses. *Blood.* 2012; 119: 2633–43.
 42. **Chiku T, Padovani D, Zhu W, et al.** H₂S biogenesis by human cystathionine γ -lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia. *J Biol Chem.* 2009; 284: 11601–12.
 43. **Ohta A, Sitkovsky M.** Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature.* 2001; 414: 916–20.
 44. **Raskovalova T, Huang X, Sitkovsky M, et al.** Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J Immunol.* 2005; 175: 4383–91.
 45. **Kopperud R, Krakstad C, Selheim F, et al.** cAMP effector mechanisms. Novel twists for an 'old' signalling system. *FEBS Lett.* 2003; 546: 121–6.
 46. **Yang Z, Day YJ, Toufektsian MC, et al.** Infarct-sparing effect of A_{2A}-adenosine receptor activation is due primarily to its action on lymphocytes. *Circulation.* 2005; 111: 2190–7.
 47. **Shryock JC, Snowdy S, Baraldi PG, et al.** A_{2A}-adenosine receptor reserve for coronary vasodilation. *Circulation.* 1998; 98: 711–8.
 48. **Grenz A, Homann D, Eltzschig HK.** Extracellular adenosine: a safety signal that dampens hypoxia-induced inflammation during ischaemia. *Antioxid Redox Signal.* 2011; 15: 2221–34.
 49. **Hedegaard ER, Nielsen BD, Mogensen S, et al.** Mechanisms involved in increased sensitivity to adenosine A_{2A} receptor activation and hypoxia-induced vasodilatation in porcine coronary arteries. *Eur J Pharmacol.* 2014; 723: 216–26.
 50. **Levring TB, Hansen AK, Nielsen BL, et al.** Activated human CD4⁺ T cells express transporters for both cysteine and cysteine. *Sci Rep.* 2012; 2: 266.
 51. **Bhatia M, Wong FL, Fu D, et al.** Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J.* 2005; 19: 623–5.