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A_1 and A_3 adenosine receptors alter glutathione status in an organ-specific manner and influence the changes after inhibition of γ-glutamylcysteine ligase

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Abstract

Adenosine levels are increased in stress and act as anti-oxidant and anti-inflammatory mediators by binding to 4 G-protein-coupled receptors. Using genetically modified mice lacking A_1 and A_3 adenosine receptors, treated with ip buthionine-[*S*,*R*]-sulphoximine injections to inhibit γ-glutamylcysteine ligase, the question was addressed whether these receptors modulate the responses to the stress related to altered glutathione levels. This study determined organ glutathione levels and expression of two sub-units of γ-glutamylcysteine ligase and the cationic x_c-transporter and found that deletion of one or both adenosine receptors influenced the responses in an organ-specific manner. The lack of A_1 and A_3 adenosine receptors is related to decreased basal glutathione content and down-regulation of γ-glutamylcysteine ligase sub-units in several organs. Moreover, responses to buthionine-[*S*,*R*]-sulphoximine were different. For example, the lack of A_3 adenosine receptors, or their blockade of A_3 by MRS 1191, caused a marked increase in gene expression, which was not observed in mice lacking both A_1 and A_3 receptors. The results indicate that A_1 and A_3 adenosine receptors play a role in antioxidant responses and their role differs in an organ-specific way.

Keywords: *A_1/A_3/A_1A_3 knockout mice, organ GSH levels, γ-glutamylcysteine ligase catalytic/modulator sub-units, x_c^- cysteine transport system, Nrf2*

Introduction

Endogenous adenosine is believed to play a key role in protecting against ischemic or hypoxic cell-injury by acting as an antioxidant and anti-inflammatory mediator at specific adenosine receptors [1,2]. Adenosine receptors are a family of G-protein coupled receptors that are ubiquitously expressed in a wide variety of tissues. This family contains four receptor sub-types: A_1 and A_3, which mediate inhibition of adenylyl cyclase; and A_{2A} and A_{2B}, which mediate stimulation of this enzyme [3–5]. Adenosine A_1 receptors (A_1ARs) are up-regulated by a variety of conditions [6] and their activation improves anti-oxidant defence [7] by reducing mitochondrial radical formation and oxidant injury [8] and by increasing cellular anti-oxidant capacity. The activation of A_3 adenosine receptors (A_3ARs) causes effects similar to that of A_1ARs by activating cellular antioxidant mechanisms and plays an anti-inflammatory role in human neutrophil-mediated tissue injury [2,9,10]. Oxidative stress can be potentially increased by enhanced production of reactive oxygen species (ROS), decreased antioxidant enzyme systems or a combination of both. Regulation of redox metabolism is mainly

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provided by the interaction between the thioredoxin (Trx) and glutathione (GSH) systems [11]. The Trx system plays a critical role in the redox regulation of protein thiols involved in signal transduction and gene regulation, whereas GSH is responsible for maintaining low redox potential and high free thiol levels in the cell. GSH, a tripeptide of γ-glutamylcysteinylglycine, is one of the most abundant antioxidants in cells and tissues and, besides being the major intracellular redox buffer in the cells, modulates ionotropic receptor function and participates in cellular signal transduction pathways through direct interaction with key cysteines at the active site/modulator regions of kinases, phosphatases and transcription factors [12,13]. GSH is synthesized in two sequential enzymatic reactions catalysed by γ-glutamylcysteine ligase (GCL, EC 6.3.2.2) and glutathione synthetase (GS, EC 6.3.2.3). Buthionine-[S,R]-sulphoximine (BSO) is a selective inhibitor of GCL. Exposure of cells to BSO inhibits GSH synthesis and causes depletion of intracellular GSH levels. Thus, BSO has been frequently used to study the role of GSH in association with oxidative stress-induced cell death [14,15]. *In vivo* models suggest that GSH is exported from the liver to the blood and is catabolized in the kidney to γ-glutamyl or cysteinylglycine. These compounds are distributed via the blood to all tissues where they may be incorporated into cells by the xc^- transport system and then used for *de novo* synthesis of GSH [16]. Therefore, the enzyme and the subunits of the xc^- cystine transport system xCT are related to the GSH antioxidant system. Mammalian organs have different levels of GSH strongly related to their susceptibility to oxidative damage. Although it has been shown that A1 and A3 adenosine receptors are involved in the up-regulation of antioxidant defence system and attenuation of lipid peroxidation, few studies have addressed the possibility that altered adenosine receptor-mediated effects might underlie specific oxidant-based disorders and the role of adenosine receptors in modulation of GSH levels remains largely undefined. In the present study, we have focused on genetically deleted mouse models to investigate the effects of A1 and A3 adenosine receptors in response to systemic decrease in GSH levels by BSO treatment.

Experimental procedures

Chemical agents

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Animal use and treatments

Wild-type (wt) C57BL/6 mice, A_1 knockout and A_3 knockout mice, generated as described [17,18], were a gift of Dr Johansson (Karolinska Institutet, Stockholm). A_1A_3 double knockout were obtained by mating double heterozygous $A_1 \pm A_3 \pm$ AR mice. All the experiments were conducted using protocols following EUR directives, approved by the Institutional Animal Care and Use Committee of Perugia University. Efforts were made to minimize animal stress/discomfort and 12 animals were used for each genotype group. Animals were maintained in 12-h light/dark cycles and fed *ad libitum*. Age-matched male mice weighing 25–35 g (12–14 weeks old) were used and injected intraperitoneally (ip) with BSO dissolved in saline solution at a dose of 6 mmol/kg of body weight [15] and sacrificed by cervical dislocation 3 h following BSO administration. The blockage of A_3 AR was obtained in wt mice with a ip injection of selective A_3 AR antagonist, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-($\pm$)-dihydropyridine-3,5 dicarboxylate (MRS1191; 1 mg/kg body weight) 15 min prior to BSO administration [19]. Control mice received vehicle only.

Glutathione determination

Fresh tissue was washed in ice-cold isotonic saline solution to remove debris and blood. Liver, kidney, heart, lung and brain tissue was washed in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) at 4°C for 10 min to remove RBC, then homogenized in phosphate-EDTA buffer. The pellet was suspended in sulphosalicylic acid on ice to remove proteins and centrifuged at 15 000 × g. The supernatant was immediately used for GSH determination with dithionitrobenzoic acid (DTNB) at 412 nm (molar extinction coefficient 13.6 mM^{-1} cm^{-1}) according to Rahman et al. [20].

Preparation of nuclear extract and Western blot for Nrf2

Fresh tissues, washed in Tris-buffered saline on ice, were homogenized in 10 mM HEPES, pH 7.9, containing 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM vanadate, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and 1 mM DTT. Preparations were then incubated on ice for 15 min and centrifuged at 14 000 × g for 3 min at 4°C. The supernatant (cytosolic extract) was collected and stored at −80°C until use. The resulting nuclear pellet, washed twice in ice-cold homogenization buffer, was resuspended in ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, containing 20% glycerol, 420 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A) for 30 min at 0°C on a rotating wheel. The nuclear extract was finally centrifuged at 14 000 × g for 10 min. Cytosolic and nuclear extracts (50 μg) were loaded on 12% SDS-PAGE and Nrf2 levels determined by western blotting using Nrf2(C-20) antibody (1:200; Santa Cruz Biotech, CA). α-tubulin (B-7) and Lamin B(H-90)

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antibodies (1:100, Santa Cruz Biotech, CA) were used as marker proteins for cytosolic and nuclear extracts. After secondary incubation in horseradish peroxidase-conjugated-anti rabbit IgG antibody (1:5000) (Amersham Bioscience, Little Chalfont, UK), the immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL, Pierce). Bands were analysed with the Bandscan software.

Real time PCR

Total RNA was isolated with TRIZOL Reagent (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Lab, Hercules, CA) with 1 μg RNA. Real time PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry. Murine primers, designed on sequences deposited in the NCBI GenBank database (Table I), were obtained from Invitrogen (Invitrogen Ltd, Paisley, UK). SYBR Green RT-PCR amplifications were carried out in a 96-well plate in a 25 μl reaction volume that contained 12.5 μl of 2x iQ™ SYBR® Green Super-Mix (Bio-Rad), 400 nm forward and reverse primers and 5–40 ng of cDNA. In each assay, no-template controls were included and each sample was run in triplicate. The thermal profile consisted of incubation at 95°C for 3 min, followed by 40 cycles of denaturation for 10 s at 95°C and an annealing/extension step of 30 s at 60°C. Mean of C_t values of the treated sample was compared to the untreated control sample. ΔC_t is the difference in C_t values derived from the target gene (in each assayed sample) and GAPDH while $\Delta\Delta C_t$ represents the difference between the paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta Ct}$. The relative mRNA expression levels for each gene were normalized against that of the GAPDH gene from sample preparation.

Data analysis

Each assay was performed at least in triplicate unless otherwise indicated. Data are expressed as means ± SD and significance was assessed by a one-way ANOVA. A p-value < 0.05 was considered significant in all cases.

Results

Changes in GSH levels in organs are associated with A_1 and A_3 adenosine receptors

Organ GSH levels reflect a dynamic balance between GSH synthesis and its utilization in the maintenance of redox homeostasis. Therefore we started our study by determining organ GSH levels and the alterations induced in liver, lung, brain, kidney and heart at 3 h after ip BSO injection as organ response in each genotype (Figure 1). In wild type mice each organ showed specific GSH levels that appeared to be maximal in liver. The BSO treatment decreased liver, kidney and heart GSH content while it did not affect other organ levels. The lack of either A_1 or A_3 adenosine receptors, i.e. A_1ko, A_3ko and A_1A_3ko mice, was associated with reduction in basal hepatic GSH content that was further decreased by BSO treatment. Other organs GSH status was differently influenced either by the absence of receptor expression or by BSO treatment. In particular, after a 3 h treatment with BSO, A_3ko lungs showed an increase in GSH levels. The results indicate that A_1 and A_3 adenosine receptors might influence either organ GSH basal status or organ-specific response to GSH depletion.

Changes in organ gene expression are associated with A_1 and A_3 adenosine receptors

γ-glutamylcysteine ligase catalytic (GCLC) and modulator (GCLM) sub-units and the sub-unit of the x_c^- cystine transport system (xCT) are related to GSH levels. To investigate the role of A_1 and A_3 adenosine receptors in the response to GSH depletion, first we determined, by real time PCR analysis, the expression of these genes, reported as the ratio between gene/GAPDH mRNA, in the organs of untreated mice (Figure 2). Liver of wild type mice showed basal gene expression that was decreased in the modified genotypes, whereas in the kidney the concomitant lack of A_1 and A_3 receptors resulted in a less marked down-regulation of the expression of the enzyme sub-units. In the brain, enzyme sub-unit expression was decreased by the lack of adenosine receptors, whereas xCT expression was increased in all modified genotypes. Pulmonary GCLC expression was particularly down-regulated in A_3ko mice whereas cardiac GCLM sub-unit expression was increased in double ko mice. These results are consistent with the notion that gene expression is organ-specific and influenced by adenosine receptors. Then we analysed the effects of the lack of A_1 and A_3 adenosine receptors in the modulation of organ-specific gene expression caused by BSO treatment, by determining fold-induction changes, assuming the corresponding untreated gene expression as 1 (Figure 3). Among the analysed genes, xCT sub-unit seemed to be strongly affected by the presence/absence of the adenosine receptors. The absence of the A_3 receptor resulted in a marked xCT up-regulation in liver, brain and lung, whereas gene cardiac expression was strongly increased in double ko mice. Among the analysed organs, the most striking differences in gene modulation were observed in the lung. In particular, GCL sub-units and xCT were markedly up-regulated in the A_3ko genotype in agreement with the increases in GSH

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Table I. List of primers.

Accession number	Gene name	Gene symbol	Primer sequences (F: forward; R: reverse)
XM983502	Glyceraldehyde-3- phosphate dehydrogenase	GAPDH	F.GCCAAATTCAACGGCACAGT R.AGATGGTGATGGGCTTCCC
NM010295	γ-glutamylcysteine ligase catalytic sub-unit	GCLC	F.GGCGATGTTCTTGAGACTCTGC R.TTCCTTCGATCATGTAACTCCC
NM008129	γ-glutamylcysteine ligase modifier sub-unit	GCLM	F.CACAGGTAAAACCCAATAGTAACCAA R.GTGAGTCAGTAGCTGTATGTCAAATT
NM011990	Cationic aminoacid transporter, x_c^- system	xCT	F.CCTGGCATTTGGACGCTACAT R.TCAGAATTGCTGTGAGCTTGCA

levels found in these animals after BSO treatment. The results suggest that A_1 and A_3 adenosine receptors influence organ-specific response to GSH depletion.

Changes in pulmonary GSH levels and gene expression after treatment with A_3 adenosine receptor antagonist

To better investigate the pulmonary response of mice lacking the A_3 adenosine receptor, we performed a time-course study of GSH levels (Figure 4A). Within the first hour, the treatment with BSO caused a marked fall in GSH levels followed by the observed increase at 3 h, showing that pulmonary GSH levels in A_3 ko animals are promptly depleted. On the contrary, lungs of wild type mice did not seem to be affected by the BSO treatment in the indicated time-interval. To determine the causal relationship between adenosine receptors and lung-specific response to systemic GSH depletion, we treated wild type mice with the A_3 selective antagonist, MRS1191, 15 min prior to BSO administration. Lung were removed and used to determine GSH levels and gene expression (Figure 4B and C). Consistent with results obtained with A_3 ko mice, BSO treatment, after an early fall in GSH status, increased lung GSH levels and increased mRNA levels of GCL sub-units and xCT. We then analysed Nrf2 nuclear translocation in lungs of genetic and pharmacological A_3ko mice (Figure 4D). In both animals, systemic GSH depletion led to nuclear Nrf2 translocation consistent with the observed gene up-regulation. Treatment of A_3ko mice with MRS1191 did not modify the responses of this genotype. Data suggest that the lack of A_3 adenosine receptor is, at least partially, responsible for the pulmonary response.

Discussion

We used an *in vivo* approach to investigate whether/how A_1 and A_3 adenosine receptors influence organ-specific GSH levels and modulate tissue responses to changes in GSH status 3 h after ip BSO treatment. The results of the present study show that: (i) the lack of A_1 and A_3 adenosine receptors is related to decreased basal GSH content and down-regulation of GCL sub-units in several organs, (ii) the lack of A_1 and A_3 adenosine receptors modulates gene expression in organ responses to systemic GSH depletion, and (iii) the lack of A_3 adenosine receptors increases lung susceptibility to systemic GSH depletion and causes a marked adaptive response. Many strategies have been used to cause GSH depletion and previous studies have demonstrated that ip injection of BSO is a valid strategy to deplete GSH levels in organs *in vivo*. In adult mice BSO is readily absorbed in liver, kidney, lung and heart and, because of the blood–brain barrier, poorly distributed to the brain [15]. GSH content reflects a dynamic system that responds to alterations in the cellular environment, such as the treatment with BSO, a GCL inhibitor [21]. Inhibition of this enzyme decreases cellular levels of GSH, since GSH continues to be exported and utilized without significant resynthesis. To date, no study has directly addressed the involvement of these two adenosine receptors, generally coupled to the $G_{i/o}$ family of G proteins, in the control of GSH status and in the modulation of expression of enzymes/proteins. A_1 and A_3 receptors are activated by basal adenosine concentration [3,4,22], therefore it is plausible that these receptors have physiological similar roles [1,2,5]. GSH is a major free radical scavenger and its depletion is generally associated with the synthesis of new GCL sub-units, i.e. a larger catalytic sub-unit (GCLC) and smaller modulator sub-unit (GCLM), that lead to an increased GSH synthesis. Its catalytic efficiency is increased by holoenzyme formation and there is a differential up-regulation of the GCL sub-units in response to cysteine deprivation through the transcription factor Nrf2, which binds to the EpRE/ARE element [22–24]. Nrf2 is a transcription factor that binds to Keap1, forming a complex that, under non-oxidizing conditions, remains sequestered in the cytosol [25–27]. Following an oxidative insult, Nrf2 dissociates from the Keap1-Nrf2 complex and translocates to the nucleus, binding to ARE, thereby potentiating the cellular response against electrophil/oxidative stress [25]. L-cystine supply is usually ensured by cystine/glutamate transporter, whose activity, mediated by xCT, is induced by various

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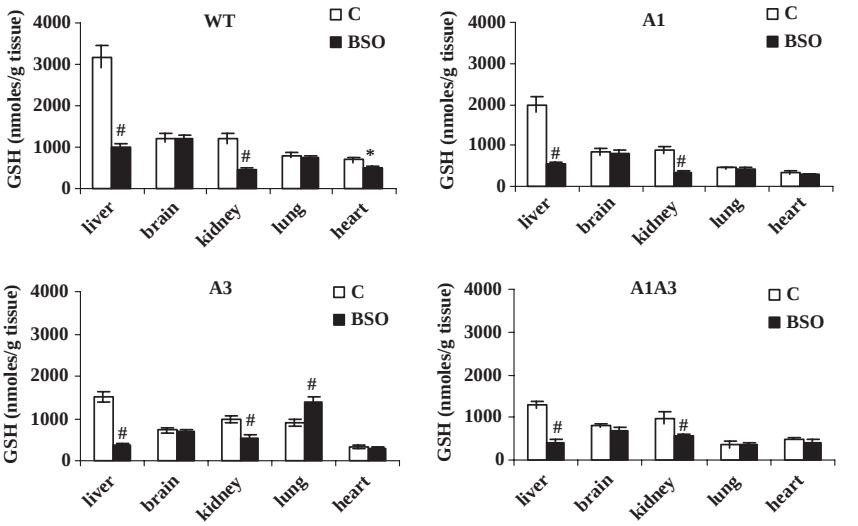

Figure 1. Organ GSH levels after BSO injection in different genotype mice. Animals were ip injected with 6 mmol/kg body weight BSO. After 3 h, organs were removed for GSH analysis as described. Control mice were injected with saline solution. Data, expressed as nmol/g tissue ± SD ($n = 6$) were analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs control.

stimuli via Nrf2 activation [16,28]. Although it is plausible to propose that the increased GCL expression, the greater availability of L-cysteine and the decreased intracellular levels of GSH will increase the rate of GSH synthesis, information on levels of substrates, enzymes and transporters will not predict level of GSH and vice versa. Nevertheless, our results showed that high/increased GSH levels are generally related to substrate availability and holoenzyme formation. High levels of expression of GCLC and

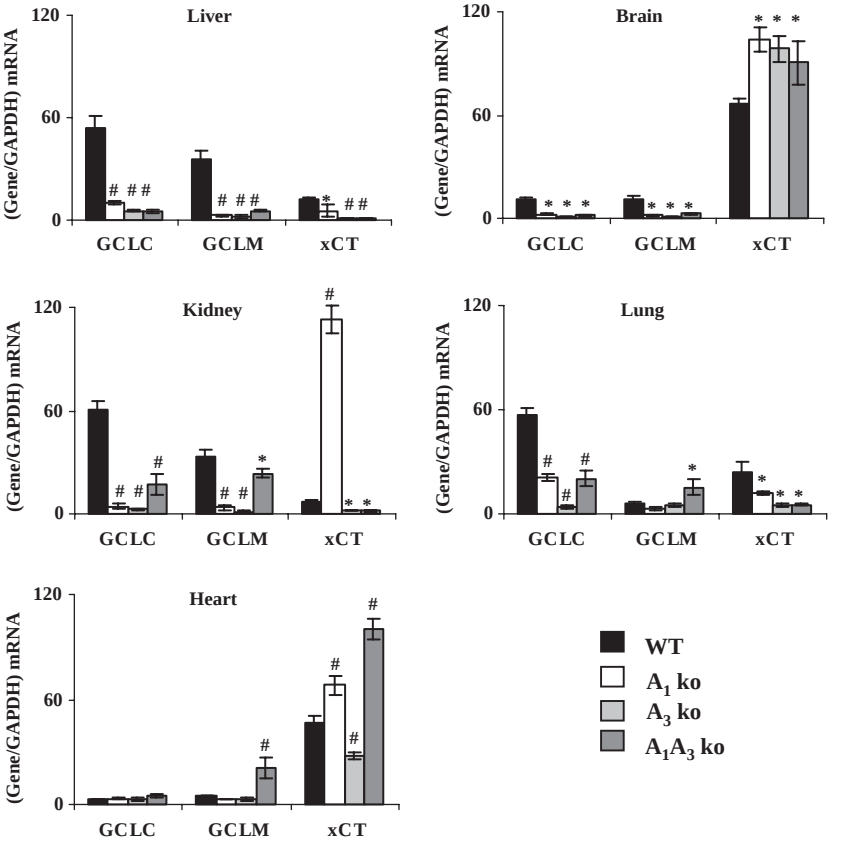

Figure 2. Organs of different genotype display specific gene expressions. Mice were ip injected with saline solutions and real time PCR performed as described. Values ($\times 10^2$ for GCLC and GCLM; $\times 10^4$ for xCT) represent the ratio between mRNA of the analysed gene and GAPDH. Results are given as mean ± SD ($n = 6$). Data were analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs wild type.

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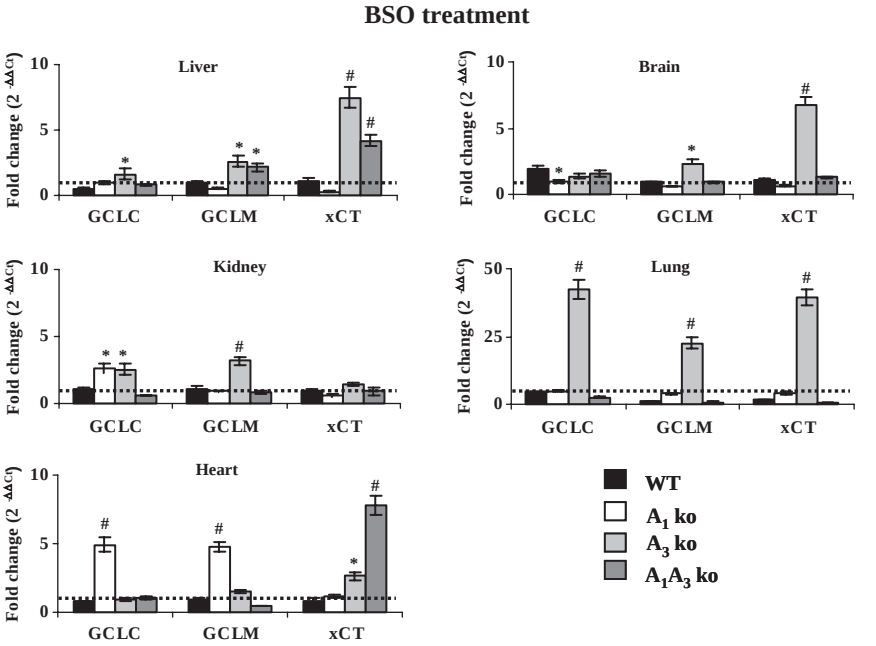

Figure 3. A_1 and A_3 adenosine receptors influence gene expression after BSO treatment. Real time PCR analyses of murine organs of different genotypes revealing changes in the expression of genes, whose values were normalized to GAPDH expression and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA abundance of each gene in untreated control organ was assumed as 1. Results are given as mean $\pm$ SD ($n=6$). Data were analysed by ANOVA. $\star p<0.005$, $\# p<0.001$ vs control.

GCLM sub-units and of xCT are consistent with the high hepatic basal GSH levels found in wild type animals and with the increases in GSH levels found in the lung of A_3ko mice after BSO treatment. Our experimental animal models, besides showing significant difference in organ-specific GSH status in mice of different genotypes, also showed the possible involvement of A_1 and A_3 receptors in the modulation of genes involved in the response to systemic GSH depletion. Because A_1 and A_3 adenosine receptor expression contributes to cellular antioxidant defence systems, all knockout murine organs, as expected, showed a decreased expression of GCL sub-units compared to wild type animals. BSO is poorly transported to the brain because of the blood–brain barrier, therefore in all the genotypes we did not find large changes in GSH levels after 3 h treatment. In basal conditions the lack of the adenosine receptors was related to a significant down-regulation of both enzyme sub-units and a strong up-regulation of xCT sub-unit. Moreover, we showed that the lack of A_3 adenosine receptor influences the sensitivity of the lung to systemic GSH depletion. After an initial fall in pulmonary GSH levels, the organ displayed a marked adaptive response through Nrf2 activation. The fact that the same response was observed in wild type mice treated with the A_3 selective antagonist, MRS1191, further supports the causal relationship between adenosine receptors and lung-specific response to GSH depletion. The mechanism by which the lack of A_3 receptor expression sensitizes the lung to GSH depletion is still to be clarified. Furthermore, the fact that the double knockout does not show the same phenotype as the A_3 knockout indicates that also A_1 receptors are very important.

Data on physiological roles of A_3 receptors are rather conflicting, since these receptors appear to have a complex role in inflammation either with pro-inflammatory or anti-inflammatory effects [19]. A_3 activation before renal ischemia results in the worsening of renal function and mice lacking A_3 receptors display improved renal function after ischemia/reperfusion injury [29]. In contrast, selective A_3 agonist, IB-MECA, reduces inflammation in murine models of colitis and reduces LPS-induced mortality [30,31]. Although the protective effects of IB-MECA against endotoxemia might be mediated by A_{2A} receptors activated by high doses of IB-MECA [32], the role of A_3 receptors in protecting against sepsis and inflammation is far from clear. Moreover, it is to note that also metabolites of adenosine may play a key role in the A_3 receptor response, since inosine, that accumulates in tissues under inflammatory conditions [33], is a highly selective agonist for A_3 receptor, suggesting that this receptor may be important in mediating pathophysiological changes [34]. However, besides the A_3 receptor involvement, to date the existence of other pulmonary factors contributing to the final effect cannot be totally disregarded.

In conclusion, our results, obtained with genetically-modified animals lacking either one or combinations of adenosine receptors, show that organ GSH status, basal gene expression and gene response to GSH depletion are possibly related to A_1 and A_3 adenosine

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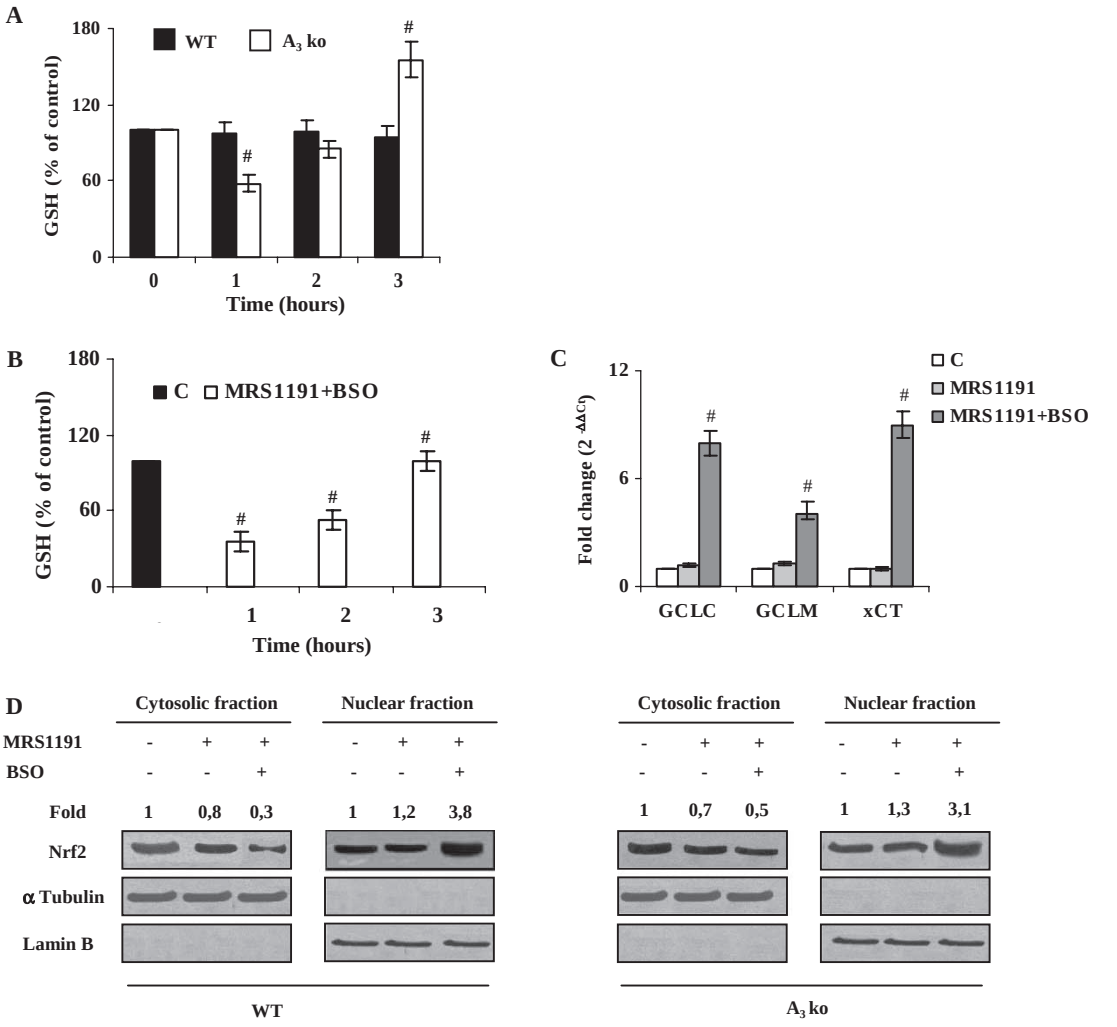

Figure 4. A_3 adenosine receptors antagonist modulates GSH levels and gene expression. (A) Time-course of GSH levels in lung of wild type and A_3 ko mice after BSO treatment. Animals were treated as described and lungs were removed at each indicated time for GSH determination. Results are given as percentages of control (mean ± SD, $n = 6$). Control values (100%) are indicated in Figure 1. Data were analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs control. (B) Wild type animals were ip injected with MRS1191 (1 mg/kg body weight) 15 min prior to treatment with BSO (6 mmol/kg body weight). Lungs were removed for GSH determinations at the indicated time. Control mice were injected with vehicle. Results are given as percentages of control (mean ± SD, $n = 6$). Control values (100%) are indicated in Figure 1. Data were analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs control. (C) Real time PCR analyses after 3 h BSO injection of lungs of MRS1191-treated mice revealing changes in the expression of genes, whose values were normalized to GAPDH expression and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA abundance of each gene in untreated animals was assumed to be 1.0 (control). Results are given as mean ± SD ($n = 6$) and analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs control. (D) Nrf2 protein in pulmonary cytosol and nuclear fractions of A_3 ko and MRS1191-treated mice. The nuclear and cytosolic extracts, containing 50 μg of proteins, were subjected to Western blotting analyses with the indicated antibodies. Anti-α-tubulin and anti-lamin B antibodies were used as markers for the cytosolic and nuclear extracts, respectively. Data, expressed as means ± SD ($n = 6$) were analysed by ANOVA. $\star p < 0.005$, $\# p < 0.001$ vs control.

receptors, suggesting that each organ of each genotype is characterized by a different relation among the analysed parameters. Moreover, results obtained in the presence of the A_3 adenosine receptor antagonist seem to reinforce the causal link between adenosine receptors and organ responses. These novel observations might be useful for future mechanistic studies.

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