CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem. Funct. **18**, 229–234 (2000)

An Alternative Parameter for Monitoring the Therapeutic Benefits of Allopurinol Simultaneously in Renal Ischaemia-Reperfusion Injury: MDA/ATP Ratio

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The therapeutic benefits of allopurinol pretreatment in renal ischaemia-reperfusion injury were investigated by monitoring renal malondialdehyde (MDA) and ATP levels together with calculated MDA/ATP ratio in ischaemic (45 min) and reperfused (15 min) rat kidneys. MDA levels remained unchanged during ischaemia, but increased after the subsequent reperfusion. ATP content of the ischaemic kidney was decreased significantly and the recovery of ATP was incomplete after the reperfusion, whereas the MDA/ATP ratio increased at both periods. Allopurinol pretreatment (40 mg kg^{-1} iv) maintained higher ATP levels during the ischaemia and inhibited the MDA formation during the reperfusion and decreased the MDA/ATP ratio at both periods. Our findings demonstrate that allopurinol exerts a biphasic protective action by preserving tissue ATP and by inhibiting lipid peroxidation during ischaemia and the reperfusion period, respectively. These findings suggest the selective involvement of two protective mechanisms in the different periods of renal ischaemia-reperfusion injury. The MDA/ATP ratio could be a useful parameter for monitoring these protective actions of allopurinol simultaneously.

KEY WORDS — adenosine triphosphate; allopurinol; kidney; ischaemia-reperfusion; malondialdehyde; MDA/ATP ratio

ABBREVIATIONS — ATP, adenosine triphosphate; MDA, malondialdehyde

INTRODUCTION

Animal models of renal ischaemia-reperfusion injury have demonstrated both the hypoxic and the oxidant components of tissue damage, which are most marked in tubules of the cortex and the outer medulla.[1] Renal ischaemia results in a rapid decrease in tissue ATP, mitochondrial damage and thereby an inability to maintain cell membrane ion gradients.[2] In addition to the lack of blood flow and oxygen delivery (ischaemia), the restoration of blood flow (reperfusion) has also been reported to contribute to renal tubular cell damage via the generation of oxygen free radicals.[3–5] Delivery of molecular oxygen to an ischaemic tissue permits xanthine oxidase to oxidise hypoxanthine and xanthine to uric acid, leading to the increased generation of hydrogen peroxide and superoxide as byproducts.[6] These free radicals cause cellular injury by attacking membrane structures through peroxidation of polyunsaturated fatty acids. The end products of this lipid peroxidation are aldehydes, such as short-chain alkanes and malondialdehyde (MDA), which may damage the critical cell and organelle membrane functions.[7] Therefore, the depletion of ATP and increase in MDA are often used as indicator parameters of renal ischaemia and reperfusion injury, respectively.

Allopurinol, a competitive inhibitor of xanthine oxidase, has been shown to have a protective effect in renal ischaemic-reperfusion injury.[3,5,8–13] Initial studies with allopurinol suggested that its protective effect is mainly due to the preservation of purine bases for the resynthesis of ATP.[14,15] More recent

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Received 7 February 2000
Accepted 21 March 2000

investigations have emphasised that the beneficial effects are due to the inhibition of oxygen free radical formation, rather than the preservation of purine bases.[3,5,10] The relative importance of these mechanisms in the protective effect of allopurinol however, has not yet been investigated systematically.

In the present study we examined the effect of allopurinol on ATP and MDA concentrations in the ischaemic and post-ischaemic periods independently in rat kidneys in order to clarify the exact mechanism involved in the protective effect of allopurinol in renal ischaemia-reperfusion injury. Furthermore, we proposed an alternative parameter, the calculated MDA/ATP ratio, for monitoring both the hypoxic and the oxidant components of renal ischaemia-reperfusion damage simultaneously in one parameter, and investigated whether this ratio might be a useful parameter for characterising the beneficial effects of allopurinol in this biphasic renal injury.

MATERIALS AND METHODS

The study was performed using male Sprague-Dawley rats weighing 175–250 g. After the rats were anaesthetised with sodium pentobarbital (60 mg kg^{-1} ip), the kidneys were exposed through a midline abdominal incision. Kidney ischaemia and reperfusion were performed as described previously.[10] Briefly, the left and right renal arteries were isolated and totally occluded with a smooth vascular clamp for 45 min. To minimise fluid loss during this period, all exposed tissues were moistened with a constant amount of Ringer's lactate solution and the abdominal incision was temporarily closed with clamps. Subsequently, the right kidney was removed and the left kidney was reperfused for 15 min before removal. The left kidney was observed after unclamping, and if visible evidence of restored blood flow was not obtained within 1 min then the animal was excluded from the study. Rats were sacrificed thereafter. Kidneys were frozen with liquid nitrogen and stored at $-70^{\circ}C$ until later analysis. A total of 50 kidneys from 30 animals were divided into five groups each containing 10 kidneys as follows. Group 1 (control): right kidneys of the rats experiencing no ischaemia and no treatment. Group 2 (ischaemia): right kidneys of the rats experiencing 45 min ischaemia, but no treatment. Group 3 (reperfusion): left kidneys of the rats experiencing 45 min ischaemia and subsequent 15 min reperfusion, but no treatment. Group 4 (treated-ischaemia): right kidneys of pretreated rats with allopurinol experiencing 45 min ischaemia. Allopurinol (40 mg kg^{-1} iv) was dissolved in 0·1 N NaOH and injected 8 min before renal artery occlusion.[5] Group 5 (treated-reperfusion): left kidneys of pretreated rats with allopurinol as above experiencing 45 min ischaemia and subsequent 15 min reperfusion.

Thiobarbituric acid and 1,1,3,3-tetraethoxypropane were purchased from Sigma Chemical Co. (St Louis, MO). Glucose-6-P dehydrogenase, hexokinase and NADP were purchased from Boehringer Mannheim (Germany). Allopurinol was purchased from Atabay (Turkey).

MDA Assay

The tissue MDA content was determined by the thiobarbituric assay as described by Uchiyama and Mihara.[16] The breakdown product of 1,1,3,3-tetraethoxypropane was used as standard and the results were expressed as nanomoles per gram of tissue.

Tissue ATP Content

The amount of kidney ATP was determined by the method based on the presence of hexokinase and glucose-6-P dehydrogenase (G6P-DH) in coupled reactions:[17,18]

$$\mathrm{ATP} + \mathrm{glucose} + \mathrm{Mg}^{+2} \xrightarrow{\text{G6P-DH}} \mathrm{glucose\text{-}6\text{-}phosphate} + \mathrm{ADP} \quad (1)$$

$$\mathrm{Glucose\text{-}6\text{-}phosphate} + \mathrm{NADP}^{+} \xrightarrow{\text{Hexokinase}} \mathrm{gluconolactone\text{-}6\text{-}phosphate} + \mathrm{NADPH} + \mathrm{H}^{+}. \quad (2)$$

The end product NADPH produced in the reaction was monitored spectophotometrically (Milton Roy Spectronic 3000) at 340 nm. The amount of ATP in the sample was expressed as micromoles of ATP per gram of tissue.

Statistical Analysis

All values presented in Figures 1A, 1B and 2 are expressed as mean ± SE. Comparison between groups was made using Student's *t*-test with Bonferroni's modification.

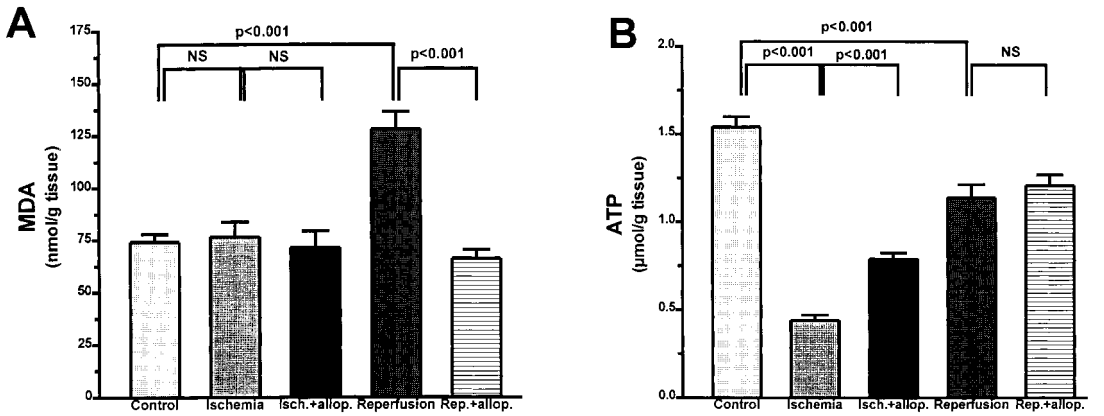

Figure 1. Effect of allopurinol, an inhibitor of xanthine oxidase, on renal MDA (A) and ATP (B) levels (mean ± SE) during 45 min ischaemia and 15 min reperfusion.

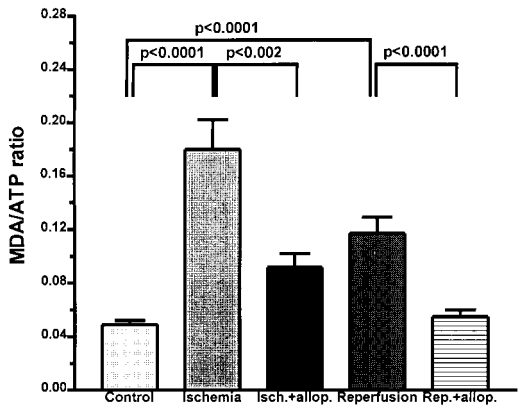

Figure 2. Effect of allopurinol, an inhibitor of xanthine oxidase, on calculated renal MDA/ATP ratio (mean ± SE) during 45 min ischaemia and 15 min reperfusion.

RESULTS AND DISCUSSION

Kidney MDA, measured after 45 min renal ischaemia, was not different from that measured in control kidneys (77 ± 7 and 74 ± 4 nmol g^{-1} tissue, respectively), but significantly increased to 128 ± 8 nmol g^{-1} tissue after 15 min reperfusion. Allopurinol had no effect on the MDA level during ischaemia, but significantly decreased the MDA level to 66 ± 4 nmol g^{-1} tissue during reperfusion (Figure 1A).

Total ATP control decreased significantly to $0{\cdot}42 \pm 0{\cdot}06$ μmol g^{-1} tissue after 45 min ischaemia, but increased to $1{\cdot}14 \pm 0{\cdot}07$ μmol g^{-1} tissue after 15 min reperfusion compared to the control value of $1{\cdot}54 \pm 0{\cdot}06$ μmol g^{-1} tissue. Allopurinol increased the ATP content to $0{\cdot}79 \pm 0{\cdot}03$ and $1{\cdot}21 \pm 0{\cdot}06$ μmol g^{-1} tissue both during ischaemia and reperfusion, respectively. The increase in the ATP content was significant in the ischaemia, but not in the reperfusion period (Figure 1B).

Forty-five min ischaemia and subsequent 15 min of reperfusion increased the MDA/ATP ratios to $0{\cdot}180 \pm 0{\cdot}022$ and $0{\cdot}117 \pm 0{\cdot}012$, respectively, as compared to the control value of $0{\cdot}049 \pm 0{\cdot}004$. Allopurinol significantly decreased the MDA/ATP ratio to $0{\cdot}092 \pm 0{\cdot}010$ and $0{\cdot}056 \pm 0{\cdot}005$ both during the ischaemia and the reperfusion, respectively (Figure 2).

Renal ischaemia results in rapid breakdown of ATP to ADP and AMP, which is further catabolised to adenosine, inosine, hypoxanthine and uric acid.[2] Xanthine dehydrogenase, the enzyme that catalyses the oxidation of hypoxanthine and xanthine to uric acid, is believed to be converted rapidly to xanthine oxidase during ischaemia.[4] During reperfusion, there is a rapid washout of the salvageable purines, and the action of xanthine oxidase, in the presence of high levels of hypoxanthine and oxygen, is thought to be the primary source of oxygen free radicals.[3–5] Generated radicals cause lipid peroxidation of cell and organelle membranes, disrupting the structural integrity and capacity for cell transport and energy metabolism. Evidence that this type of injury actually occurs in the kidney during the post-ischaemic reperfusion rests primarily on measurement of free radical-mediated lipid peroxidation products including MDA, ethane and Schiff-bases.[5,10,19–22] Consistent with the above hypothesis and previous whole kidney studies,[3–5,7,10,20,22] we have also demonstrated that MDA levels remained unchanged during 45 min renal ischaemia, but increased after the subsequent 15 min reperfusion. Additionally, we have demonstrated

that ischaemia led to a depletion of renal ATP and its recovery was incomplete after the reperfusion period.

Thus, the loss of cellular ATP and oxygen free radical-induced lipid peroxidation production seem to be strongly related phenomena in renal ischaemia-reperfusion. Therefore, experimental approaches to the amelioration of this injury have mainly focused on the prevention of free radical-induced cellular damage and use of exogenous substrates that help to maintain the level of high energy phosphometabolites in the cells.[23,24] Our study shows that these two therapeutic approaches can be achieved by means of the xanthine oxidase inhibitor, allopurinol. We demonstrate that allopurinol, when used as a pretreatment, maintains higher ATP levels during the ischaemia and inhibits the formation of lipid peroxide measured as MDA during the reperfusion period.

Considering the mechanisms underlying the protective effects of allopurinol against ischaemic damage, two possibilities are mainly discussed. These mechanisms are related to the ability of allopurinol to inhibit xanthine oxidase, thereby protecting cellular energy stores and inhibiting free radical formation.[3,9,14,15] Allopurinol was employed in several earlier studies to prevent an irreversible loss of purine nucleotides from cells in ischaemic kidney.[15] During ischaemia, degradation of purine nucleotides proceeds via hypoxanthine through an irreversible step to xanthine and uric acid. If this irreversible breakdown is prevented by allopurinol, hypoxanthine accumulates during ischaemia. This accumulated hypoxanthine would be used to restore the nucleotide pool via the salvage pathway and thereby ATP and ADP levels are partially restored. However, more recent investigations have suggested that the beneficial effects of allopurinol come from the inhibition of superoxide radical production by xanthine oxidase rather than by maintenance of cellular levels of purine nucleotides during ischaemia.[3,4,10] Our study clearly demonstrates that both mechanisms, the enhancement of purine salvage or the prevention of oxygen free radical formation, are involved in the protective effect of allopurinol in ischaemia-reperfusion injury of the kidney. However, it is important to note that these two protective actions of allopurinol are effective in the different phases of ischaemia-reperfusion injury. The enhancement of purine salvage as demonstrated by an increase in tissue ATP is involved mainly in the ischaemic phase, whereas the prevention of oxygen free radical formation as demonstrated by inhibition of MDA is the predominant action of allopurinol in the post-ischaemic-reperfusion phase.

It is well known that ATP is the most important constituent of the cell. It is involved in the provision of energy for utilisation of glucose, synthesis of fat, protein, nucleic acid and co-enzymes, contraction of muscle and membrane transport, and its cellular level also regulates many enzymes. Therefore the cellular level of ATP is of critical importance for the viability of an organ. Furthermore, the tissue level of this compound is proposed to be the indicator of the viability of an organ exposed to ischaemic damage.[15] Conversely, oxygen free radical-induced lipid peroxidation, measured as MDA, disrupts the structural integrity of the lipid bilayer. This leads to an increased cell membrane and lysosomal permeability, impairment of ion transport and of electron transfer for oxidative phosphorylation in mitochondria. Increased lysosomal permeability causes release of hydrolytic enzymes, thereby further enhancing cell injury.[7,25] Therapeutic actions that increase the tissue concentration of ATP or inhibit MDA formation are therefore required for intervening with or lessening the ischaemic-reperfusion injury. These two approaches may provide a more complete protection than either seen alone. We hereby report that allopurinol could accomplish these two therapeutic approaches in a combined manner in ischaemia-reperfusion injury. However, histologically allopurinol showed very little effect upon the morphological changes associated with ischaemia-reperfusion injury (data not shown). The possible explanation for this discrepancy could be that the greatest morphological changes which occur as result of the changes in tissue concentration of ATP or MDA due to the allopurinol pretreatment may not be most evident within 15 min after reperfusion, but after several days from the initial ischaemic injury. Support for this hypothesis is that while the beneficial effect of allopurinol in renal ischaemia-reperfusion injury was clearly demonstrated by an enhanced rate of tissue and blood oxygenation detected using near-infrared spectroscopy, no histological benefit was observed in analysed tissue samples even after 4 h reperfusion.[26]

However, other mechanisms have also been proposed to be involved in the beneficial effect of allopurinol in ischaemia-reperfusion injury. Our laboratory has reported that allopurinol may exert a protective effect in ischaemic injury by increasing reduced glutathione levels in liver[27] and Na^+ K^+

ATPase activity, a tiol-containing membrane-bound enzyme whose activity is essential for maintenance of cell viability in the kidney.[10] Allopurinol is also known to have further haemodynamic effects in addition to its cellular effect. The pretreatment of rats with allopurinol before ischaemia has been shown to elevate renal blood flow, lower renal vascular resistance and prevent erythrocyte accumulation in the medulla.[28] Allopurinol also enhances the electron transport system[29] and improves tissue oxygenation both during ischaemia and the reperfusion phase.[26] The increase in the tissue ATP demonstrated in the present study induced by allopurinol during the ischaemia period could also be explained by the aforementioned improved tissue oxygenation. Interestingly, while allopurinol increased the tissue ATP in the ischaemia period, this was not the case in the reperfusion period. The reason for this observation could be that substrates for the resynthesis of ATP are lost to the circulation and *de novo* purine synthesis is relatively slow during the initial phase of reperfusion.

We propose an alternative parameter, namely the calculated MDA/ATP ratio, in the present study to characterise the renal ischaemia-reperfusion injury and the beneficial effect of allopurinol upon this injury. By using the MDA/ATP ratio, it is possible to monitor both the hypoxic and the oxidant components of tissue damage simultaneously in one parameter. This ratio increased both in ischaemia and in the reperfusion period when compared with controls, indicating tissue injury. It is clear that both ischaemic and reperfusion periods can induce tissue injury. However, reperfusion injury may outweigh ischaemic injury in the heart and lungs.[30,31] In contrast to the other organs, renal ischaemia-reperfusion injury has been reported to be unique in the sense that there is morphological and functional damage to the kidney during the ischaemic phase itself.[11] In the present study, the MDA/ATP ratio was found to be higher in the ischaemic period than during the reperfusion period, which might suggest that ischaemic injury itself seems to have a great impact on the total kidney damage thus further supporting the above assumption. Allopurinol pretreatment reversed the increase in MDA/ATP ratio both in ischaemia and reperfusion, and decreased the MDA/ATP ratio almost to the control value in the reperfusion period, which is also suggestive of the beneficial effect of the allopurinol.

In conclusion, the present study supports previous observations of the beneficial effects of allopurinol in renal ischaemia-reperfusion injury and demonstrates that allopurinol exerts a biphasic protective action. By inhibiting xanthine oxidase activity, allopurinol preserves the nucleotide pool by preventing the irreversible degradation of these compounds at the xanthine-hypoxanthine level, thereby facilitating purine salvage for ATP resynthesis in the ischaemia period. In addition, with the same mechanism the generation of oxygen free radicals is limited during the reperfusion period. Our study further suggests that the MDA/ATP ratio could be a useful parameter for monitoring these protective actions simultaneously.

ACKNOWLEDGEMENTS

We thank Dr Oge Tascilar for excellent surgical assistance during the experiments.

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