IN VITRO STUDIES OF THE EFFECT OF THEOPHYLLINE AND/OR N-ACETYLCYSTEINE ON REDOX HOMEOSTASIS AND KETOGENESIS IN RAT HEPATOCYTES

MAŁGORZATA STEC, EWA KURZEJA, STEFAN GAWEŁ, PIOTR WARDAS1, IZABELA MACIEJEWSKA-PASZEK, AND KATARZYNA PAWŁOWSKA-GÓRAL

Department of Food and Nutrition, Medical University of Silesia in Katowice, 41-200 Sosnowiec, Poland
1 ENT Department, Medical University of Silesia in Katowice, 40-055 Katowice, Poland
mstec@sum.edu.pl

Received: December 16, 2010 Accepted: May 6, 2011

Abstract

The examinations were conducted on hepatocytes isolated by means of enzymatic method from the liver of three-month-old Wistar rats. The cells were incubated in medium with addition of theophylline and/or N-acetylcysteine. Significant changes in the activity of SOD, GPx, and GR in hepatocytes incubated in the presence of the compounds in comparison with control cells demonstrated that theophylline and/or N-acetylcysteine disturb oxidative-reductive homeostasis of the cells. Changes in concentrations of ketone bodies, resulting in disturbances of acetoacetate to β-hydroxybutyrate molar ratio, point to an unfavourable interference of theophylline into ketogenesis, which is equivalent with the disturbance of the balance between NAD$^+$ and NADH$^+$H$^+$ in hepatocytes. N-acetylcysteine simultaneously present with theophylline in incubation medium exerted a protective action on ketogenesis.

Key words: rats, hepatocytes, theophylline, N-acetylcysteine, antioxidant enzymes, ketogenesis.

The efficacy of theophylline action in bronchial asthma and chronic obstructive pulmonary disease (COPD) makes it a commonly used medication in these diseases (3-5, 13). Its action results in bronchial dilatation, moreover, it has the potential of inhibiting inflammatory reactions and acts in immunomodulative mode (8, 9, 15, 16). Despite a vast literature devoted to theophylline, not all aspects of its action in the body have been fully understood, especially those dealing with oxidative stress of the body. Oxidative stress and oxidation-reduction homeostasis disturbances, which are connected with it, are reflected in changes of cellular antioxidative enzymes activity, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and in the activity of enzymes supporting this system, particularly glutathione reductase (GR). Oxidative homeostasis is also closely connected with a variety of metabolic processes, especially those in which redox processes take place, with participation, among others, of nicotinamide adenine dinucleotide (NAD$^+$). One of the processes taking place with NAD$^+$ in the liver is the formation of ketone bodies. Evaluation of the molar ratio of oxidised to reduced nicotinamide adenine dinucleotide forms (NAD$^+/$/NADH$^+$/H$^+$) illustrates metabolic efficiency of the organ. Williamson et al. (26, 27) claimed that the molar ratio of acetoacetate to β-hydroxybutyrate formed in hepatocytes and present in arterial blood, reflects NAD$^+$ to NADH$^+$/H$^+$ molar ratio. Thus, the ratio of molar concentrations of acetoacetate to β-hydroxybutyrate in arterial blood points to energy supplies and redox homeostasis of the liver, and is known as arterial ketone body ratio (AKBR). In view of the above, one should conclude that the ratio of molar concentrations of acetoacetate to β-hydroxybutyrate, directly assayed in hepatocytes, reflects their metabolic condition and may be designated as ketone body ratio (KBR). The aim of the study was to evaluate the effects of theophylline and/or N-acetylcysteine action on isolated rat hepatocytes in the aspect of inducing oxidative stress in them and disturbing their energy balance.

Material and Methods

Hepatocytes were isolated from the liver of 3-month-old Wistar rats by the two-step collagenase perfusion method according to Berry and Friend (6), modified by Seglen (22) and Wagle (24). The cells were used if their viability was greater than 95% (assessed by trypan blue exclusion) according to Page et al. (20).

The isolated hepatocytes were resuspended in sterile Hepatocyte Medium (1 million cells per millilitre) in culture flasks (Nunclon™, Nalgene Nunc International, Denmark) and incubated for 6 h. Then, the medium was replaced. The used media contained Hepatocyte Medium and:
‐ physiological solution of sodium chloride in final concentration of 9.0 mg/dm³ (sample 1),
‐ N-acetylcysteine in final concentration of 14.4 mg/dm³ (sample 2),
‐ N-acetylcysteine in final concentration of 24.0 mg/dm³ (sample 3),
‐ theophylline in final concentration of 12.0 mg/dm³ (sample 4),
‐ theophylline in final concentration of 20.0 mg/dm³ (sample 5),
‐ N-acetylcysteine in final concentration of 14.4 mg/dm³ and theophylline in final concentration of 12.0 mg/dm³ (sample 6),
‐ N-acetylcysteine in final concentration of 24.0 mg/dm³ and theophylline in final concentration of 20.0 mg/dm³ (sample 7).

The samples were incubated in atmosphere with addition of 5% CO₂ at 37°C. The hepatocytes were incubated for 30, 60, and 120 min. Afterwards, the cells were centrifuged and homogenised with mechanical homogeniser (Ultra-Turrax T8, IKA – Laborotechnik, Germany).

In the prepared material the following parameters were assayed:
‐ content of total protein by means of Lowry method in Wang’s modification (25);
‐ activity of superoxide dismutase (SOD) with Ransod test sets (Randox, U.K.), in which assays were based on Beauchamp and Fridovich’s method (18);
‐ activity of glutathione peroxidase (GPx) with Ransel test sets (Randox), in which assays were based on Paglia and Valentine’s method (21);
‐ activity of glutathione reductase (GR) with glutathione reductase test sets (Randox), in which assays were based on Goldberg and Sponer’s method (14);
‐ total concentration of ketone bodies with Autokit Total Ketone Bodies test sets (Wako Pure Chemical Industries, Ltd., Japan);
‐ concentration of β-hydroxybutyrate with Autokit 3-HB test sets (Wako).

The concentration of acetoacetate in these samples constituted the difference between assayed total concentration of ketone bodies and concentration of β-hydroxybutyrate.

From the concentrations of acetoacetate and β-hydroxybutyrate, the value of their molar concentration ratio (ketone index – KBR) for hepatocytes incubated with the examined substances was calculated.

Activities of the examined enzymes were recalculated to 1g of cell protein.

Repeated ANOVA analyses and software STATISTICA PL v. 6.0 were used to evaluate the results of the experiments.

Results

The presence of theophylline and/or N-acetylcysteine in incubation medium of hepatocytes caused the disturbance of redox homeostasis of the cells, which was proved by changes in activity of the assayed enzymes. After 30 min of cells’ incubation with theophylline and/or N-acetylcysteine, the SOD and GPx activities decreased (with the exception of GPx activity in medium with 14.4 mg/dm³ of N-acetylcysteine) compared to control hepatocytes; however, the activity of the GR was different. After 60 and 120 min, the activities of SOD, GPx, and GR in cells incubated with theophylline and/or N-acetylcysteine were increased (Tables 1-3).

The presence of theophylline in the medium resulted in a decrease in KBR value in hepatocytes, and the presence of N-acetylcysteine increased KBR value after 30 min incubation. After 120 min of cells’ incubation with theophylline and/or N-acetylcysteine, a decrease in KBR values was demonstrated (Tables 4-5).

Discussion

In this study an attempt was made to evaluate oxidative stress of isolated rat hepatocytes treated with theophylline in the presence of recognised antioxidant N-acetylcysteine (1, 10), and evaluation of energy balance of hepatocytes under these conditions. The evaluation of hepatocyte oxidative stress was performed by determination of SOD, GPx, and GR activities after 30, 60, and 120 min incubation with the examined substances, added to medium in two different concentrations. The evaluation of cell energy efficiency was conducted by determination of acetacetate and β-hydroxybutyrate concentrations and calculation of their molar concentration ratio.

Application of isolated hepatocytes as an experimental model, with complete awareness of differences between in vitro and in vivo examinations, made it possible to observe direct effects of theophylline and/or acetylcysteine action upon cells in the aspect of activity alternations of the examined enzymes and ketogenesis intensity. The use of acetylcysteine was justified not only by its antioxidative properties, but also by its strong mucolytic action, which is used in the therapy of chronic obstructive pulmonary disease (COPD) combined with theophylline. In case of both: theophylline and acetylcysteine, hepatocytes were incubated in media containing two different concentrations of the examined substances. These concentrations are very close to concentration values in patients’ blood, observed during administration of these medications (19).

Activities of the examined enzymes in hepatocytes, as well as the concentrations of ketone bodies were referred to assays performed in control hepatocytes, isolated and incubated simultaneously with the examined cells. All three assayed enzymes interact in a particular relation. This relation may be described in the following way: decreased GR activity and decreased SOD activity should decrease GPx activity, and this results from the fact, that the product of SOD reaction, that is H₂O₂, is the substrate for GPx. The product of GR reaction, reduced glutathione (GSH), is necessary to preserve GPx activity.
Table 1
Mean values of SOD, GPx, and GR (I.U./mg protein) activities in hepatocytes after 30 min incubation with the examined substances

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substances and their concentration in medium</th>
<th>SOD (n=6)</th>
<th>GPx (n=6)</th>
<th>GR (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl 9.0 mg/dm³ (control)</td>
<td>102.19 ±6.24</td>
<td>20.29 ±0.89</td>
<td>2.21 ±0.09</td>
</tr>
<tr>
<td>2</td>
<td>N-acetylcysteine 14.4 mg/dm³</td>
<td>79.31 ±7.71*</td>
<td>23.88 ±0.82*</td>
<td>2.75 ±0.11*</td>
</tr>
<tr>
<td>3</td>
<td>N-acetylcysteine 24.0 mg/dm³</td>
<td>82.14 ±7.22*</td>
<td>19.60 ±0.70*</td>
<td>2.3 ±0.10*</td>
</tr>
<tr>
<td>4</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>91.17 ±5.22*</td>
<td>12.09 ±0.54*</td>
<td>2.11 ±0.09*</td>
</tr>
<tr>
<td>5</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>86.93 ±5.11*</td>
<td>10.59 ±0.41*</td>
<td>2.13 ±0.08*</td>
</tr>
<tr>
<td>6</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>+ N-acetylcysteine 14.4 mg/dm³</td>
<td>83.79 ±5.12*</td>
<td>17.15 ±0.84*</td>
</tr>
<tr>
<td>7</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>+ N-acetylcysteine 24.0 mg/dm³</td>
<td>98.42 ±6.97*</td>
<td>12.64 ±0.67*</td>
</tr>
</tbody>
</table>

* P<0.05; ± SD.

Table 2
Mean values of SOD, GPx, and GR (I.U./mg protein) activities in hepatocytes after 60 min incubation with the examined substances

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substances and their concentration in medium</th>
<th>SOD (n=6)</th>
<th>GPx (n=6)</th>
<th>GR (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl 9.0 mg/dm³ (control)</td>
<td>89.92 ±5.01</td>
<td>17.04 ±0.52</td>
<td>2.11 ±0.06</td>
</tr>
<tr>
<td>2</td>
<td>N-acetylcysteine 14.4 mg/dm³</td>
<td>91.20 ±7.17*</td>
<td>28.55 ±0.86*</td>
<td>2.30 ±0.07*</td>
</tr>
<tr>
<td>3</td>
<td>N-acetylcysteine 24.0 mg/dm³</td>
<td>110.27 ±4.13*</td>
<td>20.99 ±0.85*</td>
<td>2.57 ±0.07*</td>
</tr>
<tr>
<td>4</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>130.59 ±6.34*</td>
<td>19.63 ±0.89*</td>
<td>2.23 ±0.09*</td>
</tr>
<tr>
<td>5</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>91.68 ±4.75*</td>
<td>18.53 ±0.81*</td>
<td>2.34 ±0.08*</td>
</tr>
<tr>
<td>6</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>+ N-acetylcysteine 14.4 mg/dm³</td>
<td>98.29 ±4.96*</td>
<td>20.11 ±0.93*</td>
</tr>
<tr>
<td>7</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>+ N-acetylcysteine 24.0 mg/dm³</td>
<td>115.62 ±5.16*</td>
<td>22.17 ±0.92*</td>
</tr>
</tbody>
</table>

* P<0.05; ± SD.

Table 3
Mean values of SOD, GPx, and GR (I.U./mg protein) activities in hepatocytes after 120 min incubation with the examined substances

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substances and their concentration in medium</th>
<th>SOD (n=6)</th>
<th>GPx (n=6)</th>
<th>GR (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl 9.0 mg/dm³ (control)</td>
<td>97.98 ±4.82</td>
<td>16.24 ±0.68</td>
<td>2.16 ±0.08</td>
</tr>
<tr>
<td>2</td>
<td>N-acetylcysteine 14.4 mg/dm³</td>
<td>131.0 ±6.12*</td>
<td>36.72 ±0.89*</td>
<td>2.6 ±0.11*</td>
</tr>
<tr>
<td>3</td>
<td>N-acetylcysteine 24.0 mg/dm³</td>
<td>152.1 ±7.37*</td>
<td>42.79 ±0.85*</td>
<td>2.67 ±0.12*</td>
</tr>
<tr>
<td>4</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>162.09 ±7.25*</td>
<td>33.67 ±0.81*</td>
<td>2.6 ±0.12*</td>
</tr>
<tr>
<td>5</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>125.81 ±6.80*</td>
<td>30.46 ±0.89*</td>
<td>2.7 ±0.11*</td>
</tr>
<tr>
<td>6</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>+ N-acetylcysteine 14.4 mg/dm³</td>
<td>136.4 ±6.35*</td>
<td>42.29 ±0.68*</td>
</tr>
<tr>
<td>7</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>+ N-acetylcysteine 24.0 mg/dm³</td>
<td>149.98 ±7.65*</td>
<td>41.19 ±0.84*</td>
</tr>
</tbody>
</table>

* P<0.05; ± SD.

Table 4
Mean values of acetoacetate (μmol x 10⁶/cell) and β-hydroxybutyrate (μmol x 10⁶/cell) concentrations, and KBR values in hepatocytes after 30 min incubation with the examined substances

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substances and their concentration in medium</th>
<th>Acetoacetate (n=3)</th>
<th>β-hydroxybutyrate (n=3)</th>
<th>KBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl 9.0 mg/dm³ (control)</td>
<td>4.4 ±0.38</td>
<td>4.35 ±0.28</td>
<td>1.01</td>
</tr>
<tr>
<td>2</td>
<td>N-acetylcysteine 14.4 mg/dm³</td>
<td>6.62 ±0.56</td>
<td>4.31 ±0.38</td>
<td>1.53</td>
</tr>
<tr>
<td>3</td>
<td>N-acetylcysteine 24.0 mg/dm³</td>
<td>6.25 ±0.55</td>
<td>5.17 ±0.52</td>
<td>1.21</td>
</tr>
<tr>
<td>4</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>4.35 ±0.60</td>
<td>5.05 ±0.45</td>
<td>0.86</td>
</tr>
<tr>
<td>5</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>3.06 ±0.58</td>
<td>4.46 ±0.48</td>
<td>0.69</td>
</tr>
<tr>
<td>6</td>
<td>Theophylline 12.0 mg/dm³</td>
<td>+ N-acetylcysteine 14.4 mg/dm³</td>
<td>5.18 ±0.60</td>
<td>5.13 ±0.52</td>
</tr>
<tr>
<td>7</td>
<td>Theophylline 20.0 mg/dm³</td>
<td>+ N-acetylcysteine 24.0 mg/dm³</td>
<td>6.35 ±0.52</td>
<td>6.62±0.43</td>
</tr>
</tbody>
</table>

± SD.
The lack of the relations among the examined enzymes in the conducted experiment may point to the occurrence of additional mechanisms, independently regulating the activity of each of the discussed enzymes. An interesting fact was that after 30 min of incubation of hepatocytes with theophylline and/or N-acetylcysteine, regardless of concentrations, the activity of all three enzymes decreased in a lesser or greater degree, in comparison with the activity determined in control hepatocytes. The exception was GPx activity, determined in hepatocytes treated with N-acetylcysteine in lower concentration, when the enzyme activity was increasing. Upon prolongation of incubation time to 60 min, a tendency of an increase in the activity of the examined enzymes was observed in comparison to the control hepatocytes. The exception was the activity of GR in hepatocytes treated with lower concentration of acetylcysteine. Additionally, after 120 min of the experiment, enzyme activity in hepatocytes was in each case higher than in control ones. N-acetylcysteine is a well known antioxidant, acting protectively on GSH (11, 12), thus its presence in hepatocyte medium should not cause a decrease in GPx activity. Therefore, a decrease in GPx activity after 30 min of simultaneous incubation of hepatocytes with N-acetylcysteine and theophylline, could not be the result of glutathione deficiency. The only explanation of this situation was a decrease (after 30 min of the experiment) in SOD activity. A decrease in SOD activity results in a decrease in H$_2$O$_2$ concentration, and this in turn results in a decrease in the activity of GPx, the enzyme for which H$_2$O$_2$ is the substrate. Such explanation for a decrease in GPx activity in all samples after 30 min, with the exception of the samples containing only N-acetylcysteine, seems to be correct. The question concerning the cause of the decrease in SOD activity would require further studies. What is even more interesting is that SOD activity after 30 min of the experiment was decreasing in comparison with the control group in all hepatocytes, but the least in the samples containing theophylline with N-acetylcysteine in higher concentrations. After 60 min of hepatocytes incubation with acetylcysteine and/or theophylline, the activity of SOD and GR was higher than their activity determined in control hepatocytes, with the exception of hepatocytes treated with theophylline in higher concentration, where GPx activity after 60 min of hepatocytes incubation was in each case higher than in control hepatic cells. The fact worth mentioning is that GPx activity in case of N-acetylcysteine applied in both concentrations and in case of both mixtures of theophylline and acetylcysteine was markedly higher than under control conditions. After 120 min of the experiment, an increase in GPx activity in all samples of hepatocytes treated with N-acetylcysteine and/or theophylline in relation to control hepatocytes, was also more intense than an increase in SOD and GR activity, observed after the same period. Among all assayed enzymes, only GPx activity was equal or higher than control in hepatocytes incubated in medium with addition of N-acetylcysteine after 30 min of the experiment, and after 60 and 120 min. In the light of this evidence, and observation that GPx activity in hepatocytes was increasing with more intensity than SOD and GR activity, it may be concluded that glutathione peroxidase is the most significant enzyme (the first responded to oxidative stress) in the conducted experiment. Intensively increasing activity of GPx after 60 and 120 min of the experiment could be the factor responsible for an increase in SOD activity. This was probably related to a rapid removal of H$_2$O$_2$, which was the product of SOD action and its excess, not removed by GPx, would inhibit the activity of SOD. It cannot be ruled out that also a small increase of GR activity after 120 min of the experiment was the consequence of GPx activity, because efficient oxidation of reduced glutathione should be the factor activating glutathione reductase. The fact, that an increase in GR activity, after both 60 and 120 min of the experiment was small in comparison to an increase in SOD and GPx activity, could be the consequence of N-acetylcysteine presence in hepatocyte media. However, a small increase in GR activity observed also in hepatocytes incubated in the media only with theophylline would indicate the existence of additional mechanisms regulating GR activity. It is possible, that these mechanisms were related to theophylline ability to inhibit phosphodiesterase activity, responsible for removal of cAMP (8, 17, 23).

The above description of activity changes of the assayed enzymes in the conducted experiment may point to the occurrence of additional mechanisms, independently regulating the activity of each of the discussed enzymes.
enzymes suggests that these alterations were the consequence of changing concentrations of free radicals (superoxide anion, hydrogen peroxide) and glutathione, and this in turn was the consequence of N-acetylcysteine and theophylline presence in hepatocyte incubation media.

Introduction of theophylline and/or N-acetylcysteine to hepatocyte incubation media resulted in concentration changes of acetoacetate and β-hydroxybutyrate in the cells, and in consequence changes of KBR value, i.e. the value of acetoacetate to β-hydroxybutyrate molar ratio. No reports concerning assays of ketone bodies mentioned above in isolated cells or cell cultures were found in the literature. Therefore, there is no data available regarding interpretation of KBR value as an index of metabolic efficiency of isolated cells in in vitro examinations, as in the case of arterial ketone body ratio assayed in arterial blood (2, 7, 28) and metabolic efficiency of the liver in vivo examinations. In relation with the above, in the conducted experiment, the only reference may be the results of assays of ketone bodies concentrations in control hepatocytes. The fact that KBR values for control hepatocytes were very similar after 30 and 120 min of the experiment proves that control hepatocytes were completely metabolically efficient throughout the experiment. Therefore, any changes of KBR value in hepatocytes incubated in the media with addition of theophylline and/or N-acetylcysteine were the consequence of the presence of the examined compounds in the medium. These changes, with the passage of time (between 30 and 120 min of the experiment), were marked in successive samples with theophylline and N-acetylcysteine. An increase in acetoacetate concentration in hepatocytes incubated in the medium with addition of N-acetylcysteine and a mixture of theophylline with N-acetylcysteine in higher concentrations, after 30 min of the experiment, suggests not only the intensification of ketogenesis, but first of all, the shift of equilibrium of acetoacetate to β-hydroxybutyrate reduction reaction for the benefit of acetoacetate. Since reaction equilibrium between acetoacetate and β-hydroxybutyrate is controlled by NAD+ to NADH+H+ molar ratio, it may be concluded that in the described situation NAD+ concentration was increasing. After 120 min of the experiment, in hepatocytes incubated with addition of N-acetylcysteine, the concentration of acetoacetate was also increasing and, simultaneously, an oxidised form of NAD+. In consequence, the presence of N-acetylcysteine in hepatocyte medium resulted in an increase in KBR value after 30 min of their incubation. Assuming that KBR value is the measure of hepatocytes metabolic efficiency, it may be concluded that N-acetylcysteine increased the efficiency of these cells. It was of a particularly great significance when the presence of theophylline in the medium resulted in a decrease in acetoacetate concentration. It acts inversely to N-acetylcysteine causing, in consequence, a significant decrease in KBR value, especially in its higher concentration. Changes of ketone body concentrations point to an unfavourable interference of theophylline into ketogenesis, which is equivalent with the disturbance of equilibrium between NAD+ and NADH+H+ in hepatocytes. It was confirmed by very low KBR value in hepatocytes incubated in the medium with addition of theophylline. N-acetylcysteine present simultaneously with theophylline in incubation medium exerted a protective action on ketogenesis, which was proved by ketone body concentrations and KBR value. The activity of GPx pointed to the protective action of N-acetylcysteine in relation to this enzyme, which probably involved the interference into metabolism of glutathione.

The disturbance of oxidative equilibrium in the isolated hepatocytes culture in vitro and the disturbance of their energy equilibrium under the impact of theophylline and N-acetylcysteine, may suggest the occurrence of similar changes when both compounds are applied to living organisms. However, in vivo, these changes may be too subtle to be detected.

References


