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INTRACELLULAR REDUCED GLUTATHIONE CONTENT IN NORMAL AND TYPE 2 DIABETIC ERYTHROCYTES: EFFECT OF INSULIN AND (−)EPICATECHIN

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Catechins, polyphenolic compounds belonging to flavanoid family, have been reported to posses insulin-like properties and their antidiabetic actions have also been documented. Recently catechins have received much attention as strong anti-oxidative agents. Since oxidative stress has been implicated in the development of diabetic complications and GSH plays an important role in protection against oxidative damages, we have studied the in vitro effect of (−)epicatechin and insulin on the reduced glutathione content in normal and type 2 diabetic erythrocytes. The GSH content was significantly lower (p < 0.001) in type 2 diabetic patients as compared to normal individuals. In vitro insulin treatment (10−9 M) resulted in increase in the GSH content in both normal and type 2 diabetic erythrocytes. (−)Epicatechin (1mM) also resulted in an increase in erythrocyte GSH content in both normal and type 2 diabetic erythrocytes. Insulin gave a pronounced dose-responsive effect: maximum increase in GSH content at physiological hormone concentration and a lower increase at higher and lower insulin concentrations. (−)Epicatechin did not show a similar dose-responsive effect. Although the exact mechanism by which (−)epicatechin causes elevation of erythrocyte GSH is not clear nevertheless this finding may have important therapeutic implications. A higher content of dietary flavanoids may thus protect diabetic patients against long-term complications.

Key words: erythrocyte, glutathione, (−)epicatechin, insulin.

INTRODUCTION

The aqueous extract from the bark of Pterocarpus marsupium has been used for centuries as an antidiabetic drug in Indian folk medicine. Epicatechin, the active principle isolated from the bark of Pterocarpus marsupium, has been shown to posses insulin-like activity (1). Epicatechin is a member of a group of polyphenolic compounds collectively known as catechins present in tea, belonging to flavanoid family. Scientific studies have also emphasized the antidiabetic property of catechins in alloxan-induced diabetes (2, 3).
Reduced glutathione (GSH) is one of the major constituents of erythrocytes and plays an important role in providing protection against oxidative damages. The antioxidant function of the tripeptide is related to oxidation of the thiol group of its cysteine residue with formation of a disulfide (GSSG). Diabetic state has been shown to be associated with an increased oxidative stress (4), the antioxidant deficiency in erythrocytes has been reported to be one of the first alterations as a result of hyperglycemia even before the development of frank diabetic state (5). Evidence has accumulated that oxidative stress may play an important role in the etiology of diabetic complications (6–8).

We have studied the in vitro effect of (−)-epicatechin on the level of glutathione in erythrocytes of normal and type 2 diabetic patients. We have compared the effect of (−)-epicatechin with insulin in view of previous reports of an insulin-like activity of (−)-epicatechin on erythrocyte osmotic fragility (9) and membrane bound Na/K-ATPase (10).

MATERIAL AND METHODS

The criterion for selection of type 2 diabetic subjects was the same as reported earlier (10). Blood from 24 type 2 diabetic (14 men, 10 women) patients was taken. None of the patients had high blood pressure or microalbuminuria. Care was also taken to exclude patients who had a family history of hypertension. The mean age of type 2 diabetic patients was 56±8 years and their mean duration of diabetes was 10±4 years. Fasting blood glucose values of these subjects was 206±60 mg/100 ml and their BMI was 23±1.9 kg/m².

The control group consisted of 18 healthy volunteers (10 men, 8 women) mean age 45±9 years and the fasting glucose level was 74±12 mg/100 ml. The BMI of the normal subjects were 22.1±2.2 kg/m². None of the control were affected by hypertension. Care was taken to select only those control subjects with no family history of diabetes mellitus or hypertension (two generations). None of the women studied were receiving any hormonal treatment.

Venous blood was collected from control and type 2 diabetic patients after an overnight fasting, with informed consent, using ACD as anticoagulant. The blood samples were kept at 37°C for 3 hours prior to experiments for degradation of endogenous insulin. The blood sample was centrifuged at 4°C for 10 min. at 100 g to remove plasma and buffy coat and the isolated erythrocytes were washed 4 to 5 times with 0.154 M NaCl. Erythrocyte GSH was measured following the method of Beutler (11). The method is based on the property of GSH to reduce 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) forming a yellow coloured anionic product whose optical density is measured at 412 nm. A molar extinction coefficient of 13,600 was used for the nitrobenzoate ion. Concentration of GSH is expressed in micro moles (µM) per gram of hemoglobin.

In vitro experiments were carried out by adding bovine insulin or (−)-epicatechin to whole blood and incubating at 37°C for 30 min. prior to GSH determination. In parallel controls experiments blood was incubated without insulin or (−)-epicatechin.

Blood glucose values were determined by using Ames Glucometer GX (Miles, India). Statistical analysis was carried out by employing Student's 't' test and P < 0.01 was used as a level of significance.
RESULTS

The GSH content in the erythrocyte of normal and type 2 diabetic subjects is shown in Table. The GSH content in the erythrocyte of type 2 diabetic patients was lower as compared to controls. In vitro insulin treatment ($10^{-9} \text{ M}$) of erythrocyte resulted in increase of GSH content in both normal and type 2 diabetic subjects.

![Graph showing the dose-responsive effect of insulin on the intracellular reduced glutathione in normal and type 2 diabetic erythrocytes.](image)

*Fig. 1. Dose-responsive effect of insulin on the intracellular reduced glutathione in normal and type 2 diabetic erythrocytes (mean ± S.D. of 5–6 independent experiments).*

*Figure 1 shows the dose-responsive effect of insulin ($10^{-7} - 10^{-11} \text{ M}$) on erythrocyte GSH level. The pattern of dose-responsive insulin effect was similar in both normal and type 2 diabetic patients. Maximum increase in GSH content is observed at $10^{-9} \text{ M}$ hormone concentration, a lower increase in erythrocyte GSH content is observed at both higher and lower insulin concentrations.*

Treatment with ($-)$epicatechin (1mM) also resulted in an increase in erythrocyte GSH content in both normal and type 2 diabetic patients, an effect which was similar as that observed with insulin (Table 1). In contrast to insulin, ($-)$epicatechin did not show any dose-dependent effect on erythrocyte GSH content (data not shown). No significant effect of ($-)$epicatechin was observed below 1mM, however, a significant and similar insulin like effect was observed at both 1mM and 10mM concentrations.
Table 1. *In vitro* effect of insulin and (-)epicatechin on the erythrocyte reduced glutathione content in normal and type 2 diabetic subjects (values are mean ± S.D. of 5—6 independent experiments).

<table>
<thead>
<tr>
<th>Condition</th>
<th>GSH content*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal value</td>
<td>+insulin (10⁻⁹ M)</td>
<td>+epicatechin (1 mM)</td>
</tr>
<tr>
<td>Normal</td>
<td>6.35 ± 0.28</td>
<td>6.99 ± 0.18*</td>
<td>6.96 ± 0.21*</td>
</tr>
<tr>
<td>Type 2 diabetic</td>
<td>4.64 ± 0.29**</td>
<td>5.43 ± 0.24*</td>
<td>5.30 ± 0.23*</td>
</tr>
</tbody>
</table>

* Reduced glutathione concentration as μmole per gram hemoglobin at 37°C.
** p < 0.001 as compared to normal.
* p < 0.01 as compared to the respective control.

**DISCUSSION**

Glutathione is an important intracellular peptide with multiple functions ranging from antioxidant defense to modulation of cell proliferation (12). Our observation of a decrease in the level of erythrocyte GSH in type 2 diabetes confirms previous findings (13, 14). It has been suggested that the hyperglycemia-induced enhanced activity of the polyol pathway leads to GSH depletion, which creates a redox imbalance in diabetic erythrocytes resulting in lower defense against oxidative stress. Sustained oxidative insult causes lipid peroxidation on erythrocyte membrane, which leads to accumulation of malondialdehyde, a stable end product of lipid peroxidation (15). These alterations have been implicated in the development of long-term complications in diabetes (15—17).

The partial increase in the erythrocyte GSH content on *in vitro* treatment with physiological concentration of insulin could be due to the effect of hormone on erythrocyte glucose metabolism. Insulin has been reported to increase the capacity of erythrocytes to metabolize glucose leading to enhanced production of lactic acid and CO₂ (18). This action of insulin on glucose metabolism may help in elevating intracellular NADPH/NADP ratio thereby causing an increase in GSH/GSSG ratio.

The dose response of insulin effect on erythrocyte GSH shows a behavior typical of other insulin responses: a maximum in the physiological range (10⁻⁹ M) and relatively smaller effects at higher and lower hormone concentrations. This biphasic behavior has been found for other insulin effects on erythrocytes such as inhibition of Na/K-ATPase (10), stimulation of Na/H antiport (19), and protection of osmotic fragility (9). The lack of any dose-responsive effect of (-)epicatechin on intracellular GSH content may signify that the action of (-)epicatechin is not receptor mediated.
Our observation of an insulin-like effect of (-)-epicatechin, a flavanoid, in elevating intracellular GSH content in both normal and type 2 diabetic erythrocytes, is an interesting finding. Flavanoids are antioxidant polyphenolic compounds ubiquitously found in plants particularly onions and tomatoes, and in beverages such as tea and red wine. Recent interest in catechins has been focussed on their ability to act as natural antioxidants (20, 21). Although the exact mechanism by which (-)-epicatechin causes elevation of erythrocyte GSH content is not clear nevertheless the finding may have important therapeutic implications in view of increasing evidence that oxidative stress may be the underlying cause for most diabetic complications (7). A high content of dietary flavonoids may thus protect diabetic patients against long-term complications.

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