Research Communication

Microsomal pre-receptor cortisol production is inhibited by resveratrol and epigallocatechin gallate through different mechanisms

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Abstract

Local activation of cortisol in hormone target tissues is a major determinant of glucocorticoid effect. Disorders in this peripheral cortisol metabolism play an important role in the development of metabolic diseases, such as obesity or type 2 diabetes mellitus. Hence, dietary factors influencing the activity of the involved enzymes can have major impacts on the risk of the above diseases. Resveratrol and epigallocatechin gallate (EGCG), two natural polyphenols found in several nutriments and in green tea, respectively, are well-known for their antiobesity and antidiabetic activities. EGCG has been shown to interfere with microsomal cortisol production through decreasing the luminal NADPH:NADP$^+$ ratio. The aim of this study was to clarify if resveratrol also induces such a redox shift or causes any direct enzyme inhibition that influences local cortisol production. Cortisone–cortisol conversions and changes in NADPH levels were monitored in rat liver microsomal vesicles. Cortisol production was inhibited by resveratrol in a concentration dependent manner while the intrinsic reducing and oxidizing capacity as well as the NADPH level inside the ER-derived vesicles remained unaffected. Activity measurements performed in permeabilized microsomes confirmed that resveratrol, unlike EGCG, inhibits 11β-hydroxysteroid dehydrogenase type 1 directly. Long-term moderation of pre-receptor cortisol production likely contributes to the beneficial health effects of both polyphenols. © 2018 BioFactors, 45(2):236–243, 2019

1. Introduction

Glucocorticoids play a central role in several physiological processes including regulation of salt-water balance and intermediary metabolism, immune, and anti-inflammatory responses, reproductive processes, control of blood pressure, and mental functions [1]. Precisely regulated levels of the major stress hormone cortisol are essential for adapting to physical and emotional stress, including prolonged starvation [2]. Cortisol synthesis and secretion, adjusted to the nutrient supply and energy demands are fundamentally controlled by the hypothalamic–pituitary–adrenal axis. However, the activity of the hormone is also regulated at a pre-receptor level. Type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) is an NADP(H)-dependent enzyme localized in the lumen of the endoplasmic reticulum (ER) in glucocorticoid target tissues (liver, skeletal muscle and adipose tissue). It modulates glucocorticoid

receptor activation by producing cortisol from its inactive pro-hormone cortisone [3,4]. The NADPH cofactor for the reaction is provided by hexose 6-phosphate dehydrogenase (H6PD) [5], which is colocalized with 11βHSD1 in the ER. The substrate of H6PD, glucose 6-phosphate enters the ER lumen through a selective transporter glucose 6-phosphate translocase (G6PT) [6]. Therefore, the pre-receptor cortisol production is due to a concerted action of the G6PT-H6PD-11βHSD1 triad using a luminal NADP$^+$-NADPH pool, and it is fueled by a hexose 6-P supply from the cytosol.

In the past few years, peripheral cortisol effect has been proven to be one of the important factors in the pathomechanism of the metabolic syndrome and type 2 diabetes mellitus [7]. The role of H6PD and 11βHSD1 in these obesity-related metabolic diseases is supported by *in vivo* studies using transgenic and knockout animals [8–10]. It is therefore evident that dietary factors or medicines influencing the activity of these enzymes may have a major impact on the development of these metabolic disorders [11]. The G6PT-H6PD-11βHSD1 triad is a possible molecular target of anti-diabetic nutrition factors (e.g., active substances of green tea, red wine, chocolate etc.) of well-known beneficial health effects [12,13].

Resveratrol (3, 5, 4′-trihydroxy-stilbene) is a polyphenolic ingredient of a great variety of food (mostly grape, red wine, blueberry, peanuts, dark chocolate etc.). The biological actions of resveratrol received an increasing scientific interest in the past two decades, and a large amount of data supports its various beneficial health effects (for a review see [14]), including its anti-diabetic property [15,16]. In diabetic rat model, the polyphenol reduces blood glucose level by enhancing the cellular uptake of glucose and improving insulin action [17], thus resveratrol improves glucose tolerance while decreasing insulin resistance [18].

Evidence has been presented that the tea flavanol epigallocatechin-3-gallate (EGCG) can influence pre-receptor cortisol metabolism by causing an oxidative shift in the NADPH-NADP$^+$ pool of the ER [12]. The aim of the present study was to elucidate whether resveratrol affects hepatic microsomal cortisol production through a similar mechanism. The effect of resveratrol on the G6PT-H6PD-11βHSD1 triad was investigated by using intact and permeabilized rat liver microsomes.

2. Materials and methods

2.1. Materials

Alamethicin, cortisone, cortisol, epigallocatechin-3-gallate (EGCG), G6P, metyrapone, NADP$^+$, NADPH, 4-morpholinepropanesulfonic acid (MOPS) and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were of analytical grade.

2.2. Preparation of rat liver microsomes

Rat liver microsomes were prepared from male Wistar rats (200–250 g body weights) using fractional centrifugation [19]. Microsomes resuspended in MOPS-KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM MgCl$_2$, and 20 mM MOPS, pH 7.2) were rapidly frozen and stored in liquid N$_2$ until used. Intactness of microsomal vesicles was checked by measuring the latency of mannose 6-phosphatase activity [20], which was greater than 92% in all the preparations employed. Protein concentration of the microsomes was determined with Bio-Rad protein assay solution using bovine serum albumin as a standard according to the manufacturer's instructions.

2.3. Microsomal cortisone-cortisol conversion

G6P-driven reduction of cortisone to cortisol (i.e., the overall activity of the G6PT-H6PD-11βHSD1 triad) was measured by incubating intact microsomes (0.5 mg/ml protein concentration) in MOPS-KCl buffer with 5 μM cortisone and 50 μM G6P in a final volume of 150 μl at 37 °C for 30 min. The activity of 11βHSD1 alone was assessed directly by measuring NADP$^+$-driven cortisol oxidation under similar conditions after pretreatment of microsomes with the pore-forming alamethicin (0.1 mg/mg protein). These permeabilized vesicles (0.5 mg/ml protein) were incubated in MOPS-KCl buffer containing 5 μM cortisol and 50 μM NADP$^+$ in a final volume of 150 μl at 37 °C for 30 min. In either case, the reaction was terminated with 150 μl ice-cold methanol, and the samples were stored at −20 °C until HPLC analysis of cortisone and cortisol contents.

2.4. Fluorimetric detection of NADPH, measurement of H6PD, and 11βHSD1 activities

NADPH content of intact microsomal vesicles was monitored by continuous spectrofluorimetry using a Cary Eclipse spectrofluorimeter (Varian, Woburn, MA) at 1 mg/ml protein concentration in MOPS-KCl buffer at 22 °C temperature. Oxidation and reduction of the luminal pyridine nucleotide pool was induced by metyrapone (5 μM) and G6P (100 μM), respectively. EGCG and resveratrol were administered at 100 μM concentration. Changes in NADPH-specific fluorescence were detected at 340 nm excitation and 460 nm emission wavelengths. When resveratrol was used, the interference was minimized by setting 350 and 520 nm wavelengths.

H6PD activity was assessed by real-time fluorimetric detection of NADPH production. Intact microsomes (1 mg/ml protein) were incubated in a fluorimetric cuvette in MOPS-KCl buffer containing 2 mM NADP$^+$ and 100 μM G6P at 37 °C. The reaction was initiated after recording a stable baseline by adding alamethicin (0.1 mg/mg protein) to eliminate the membrane barrier and thus allow free access of the substrates to the intraluminal enzyme of latent activity. 11βHSD1 activity was measured similarly using cortisol (100 μM) as a substrate instead of G6P in the incubation medium. NADPH standard (10 μM) was added for calibration at the end of each fluorimetric detection in both cases.

2.5. Endogenous reducing/oxidizing capacity of the microsomal vesicles

Intrinsic cortisone reducing or cortisol oxidizing capacity of the microsomes was analyzed by incubating intact microsomal vesicles (0.5 mg/ml protein concentration) in MOPS-KCl buffer at

37 °C for 3 h in the presence of 10 μM cortisone or cortisol, respectively. The reactions were terminated by adding equal volume of ice-cold methanol. Samples were stored at −20 °C until HPLC analysis of cortisone and cortisol contents.

2.6. HPLC analysis

The methanol-treated samples were centrifuged (20,000 x g for 10 min at 4 °C) and the protein-free supernatants were analyzed to measure cortisone and cortisol simultaneously. Compounds were separated by HPLC (Alliance 2690; Waters Corp., Milford, MA) in a Nucleosil 100 C18 column (5 μm, 25 x 0.46) (Teknokroma, Barcelona, Spain) with isocratic methanol–water (58:42, vol/vol) at 0.7 ml/min flow rate; and the absorbance in the eluate was detected at 245 nm wavelength (Waters Dual λ Absorbance Detector 2487). Retention times of cortisone and cortisol were determined by injecting 10 μM standards.

2.7. Statistical analysis

Experiments were performed in triplicates, with each of the values of a single set of experiments corresponding to the mean of a minimum of 2–3 determinations. Data are presented as means + standard deviation (SD). The results were analyzed by one-way ANOVA and Tukey–Kramer Multiple Comparison Test using GraphPad Prism® software. A P value below 0.01 or 0.0001 (as indicated in figure captions) was considered as a significant difference.

3. Results

3.1. Inhibition of microsomal cortisol production by resveratrol

Effect of resveratrol on the overall activity of the G6PT-H6PD-11βHSD1 triad of the ER was tested first by using intact microsomal vesicles. The rate of cortisol production was determined
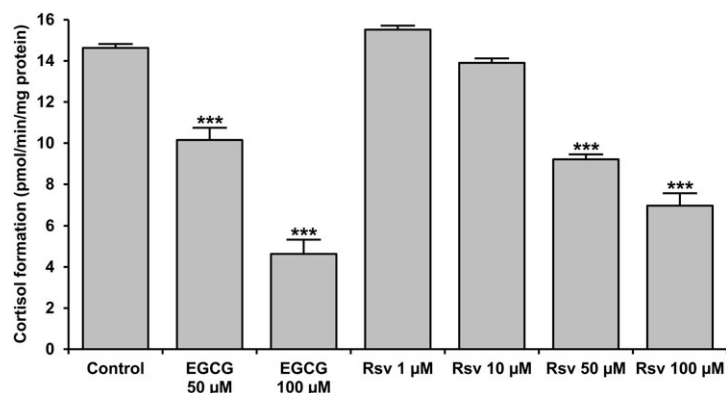

FIG 1

*Microsomal cortisol production. Intact rat liver microsomal vesicles (0.5 mg/mL) were incubated at 37°C for 30 min in MOPS-KCl buffer supplemented with cortisone (10 μM), glucose 6-phosphate (50 μM) and EGCG or resveratrol (Rsv) at different concentrations as indicated. The amount of cortisol was then measured with HPLC. The rate of cortisol production was calculated and shown as mean ± SD, n = 3, ***P < 0.0001 versus control.*

by incubating the microsomes (0.5 mg/mL) at 37 °C for 30 min in MOPS-KCl buffer supplemented with substrates: cortisone (10 μM) and glucose-6-phosphate (50 μM). Cortisol production was inhibited significantly by resveratrol when administered above 1 μM levels. The effect was concentration-dependent, and a more than 50% decrease in the activity was achieved by resveratrol at 100 μM (Fig. 1). This effectiveness was well-comparable with that of the green tea flavanol EGCG, which is a known natural inhibitor of the cortisone reducing machinery, and hence it was used as a positive control in our experiments (Fig. 1).

3.2. Luminal pyridine dinucleotide redox status

The observed inhibition of cortisol production can be due to a direct inhibition of the enzymes involved, or – as demonstrated earlier in case of EGCG – to an oxidative shift in the redox state of microsomal pyridine dinucleotides. The possible change in NADPH content of the ER vesicles can be assessed directly by fluorimetry. Luminal NADPH can be detected as a stable fluorescent baseline in intact microsomal vesicles, and this fluorescence can be efficiently depressed by the addition of cortisone, metyrapone or EGCG. The phenomenon reflects the oxidation of NADPH to NADP$^+$ as evidenced by the partial recovery of the signal upon addition of G6P (Fig. 2, upper traces). In contrast, addition of resveratrol, even at high (100 μM) concentration, did not decrease the level of NADPH. A small increase in fluorescence was seen, which might be due to an optical interference. However, the subsequent addition of G6P did not cause any further increase in the signal, which clearly shows the lack of resveratrol-induced oxidation of intravesicular NADPH to NADP$^+$ (Fig. 2, bottom trace).

Luminal NADP$^+$ and NADPH levels can also be investigated indirectly by measuring the endogenous reducing and oxidizing capacities, that is, the ability of the vesicles alone to interconvert cortisone and cortisol. This approach exploits the luminal 11βHSD1 activity, which is driven only by the intrinsic NADPH or NADP$^+$ resources in the lack of any added substrates that would provide with reducing or oxidative power. Intact microsomes were incubated with either cortisone or cortisol and the rate of cortisol or cortisone production (driven merely by endogenous reducing or oxidizing power) was measured by HPLC. This approach was successfully applied earlier to demonstrate the redox shift caused by EGCG [12] or the effects of nutritional state *in vivo* [21]. In a striking contrast to EGCG, which abolished the reducing capacity while remarkably increased the oxidizing capacity of rat liver microsomes [12], resveratrol caused an almost equally small (10–20%) decrease in both converting capacities (Fig. 3), which indicates an inhibition of 11βHSD1 rather than an alteration of NADPH:NADP$^+$ ratio in any direction. The apparently higher inhibition of oxidizing capacity in case of 100 μM resveratrol is most likely due to the general antioxidant property of the substance, which inhibits all natural oxidative processes, during the 2 h' experiment.
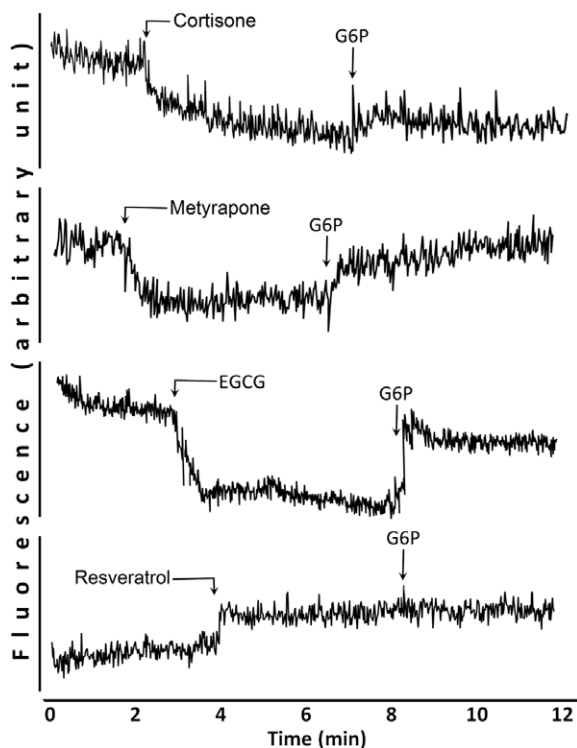
3.3. H6PD and 11βHSD1 activities

As it became evident that the obstruction of microsomal cortisol production is not due to a redox shift in the intravesicular NADPH:NADP$^+$ pool, the putative inhibition of two enzymes involved in the process was tested. Permeabilization of the microsomal membranes by the pore forming antibiotic alamethicin (0.1 mg/mg protein) uncouples H6PD and 11βHSD1 activities, and it provides the enzymes with free access to pyridine dinucleotide cofactors added to the incubation medium. Therefore, the activities of H6PD and 11βHSD1 can be measured separately in permeabilized microsomes by real time fluorescent detection of G6P- or cortisol-dependent NADPH production.

Addition of the substrate, G6P or cortisol to intact rat liver microsomes in the presence of NADP$^+$ did not trigger the formation of NADPH because the activity of both luminal enzymes, H6PD and 11βHSD1 is rendered completely latent by impermeability of the ER membrane to pyridine dinucleotides (Figs. 4
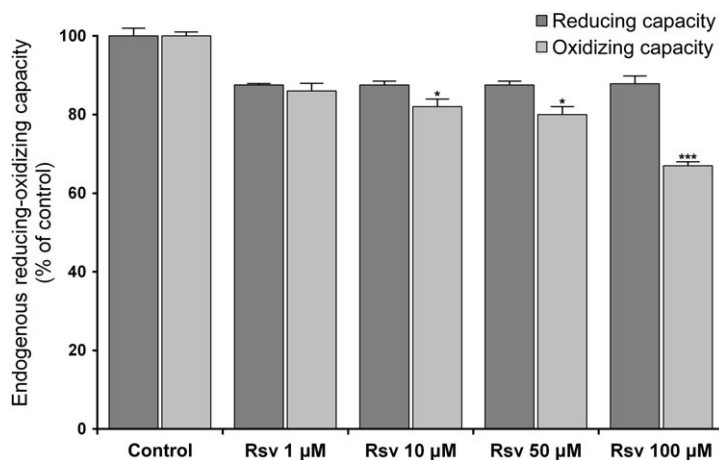
and 5). The lack of G6P- and cortisol-dependent NADPH production in intact microsomes provides additional evidence for membrane integrity and for the absence of potentially interfering extravesicular activities in the microsome preparation. A linear increase in the fluorescent signal, which corresponds to an H6PD or 11βHSD1-mediated NADPH generation started as soon as the membranes were permeabilized by alamethicin. H6PD activity was not affected significantly by resveratrol (4.62 ± 0.18 vs. 4.00 ± 0.39 nmol/min/mg protein in control and treated microsomes, respectively) (Fig. 4), while 11βHSD1 activity was inhibited in a concentration dependent manner (0.83 ± 0.15, 0.37 ± 0.09 and 0.18 ± 0.04 nmol/min/mg protein

FIG 6 *Cortisol oxidation in permeabilized microsomes 11βHSD1 activity was assessed separately on the basis of cortisol-to-cortisone conversion. Microsomal vesicles (0.5 mg/mL protein) permeabilized with alamethicin (0.1 mg/mg protein) were incubated at 37°C for 30 min in MOPS-KCl buffer supplemented with cortisol (5 μM), NADP+ (50 μM) and resveratrol in different concentrations as indicated. The amount of cortisone was then measured with HPLC. The rate of cortisone production was calculated and shown as mean $\pm$ SD, n = 3, ***P < 0.0001 versus control.*

in cases of 10, 50, and 100 μM resveratrol, respectively, vs. 0.82 ± 0.19 nmol/min/mg protein as control) (Fig. 5). This effect was also confirmed in different conditions by HPLC measurement of cortisol-to-cortisone conversion in permeabilized microsomes (Fig. 6). In line with the fluorimetric experiments, a remarkable and concentration-dependent inhibition of 11βHSD1 activity was observed. In permeabilized microsomes at close to physiological NADP+ levels, the effect of resveratrol was significant already at 10 μM concentration, and 11βHSD1 activity was almost abolished when the polyphenol was administered at 100 μM concentration.
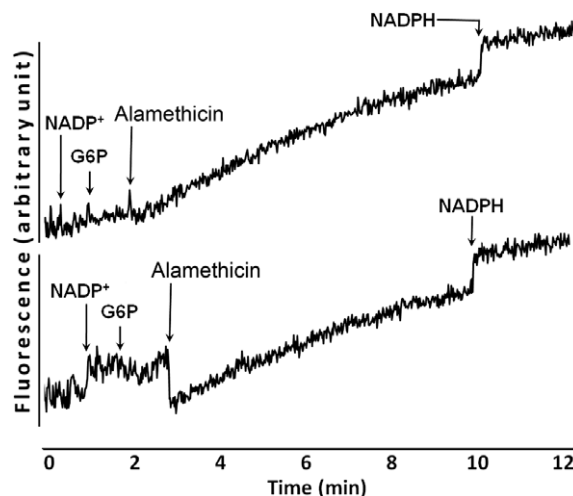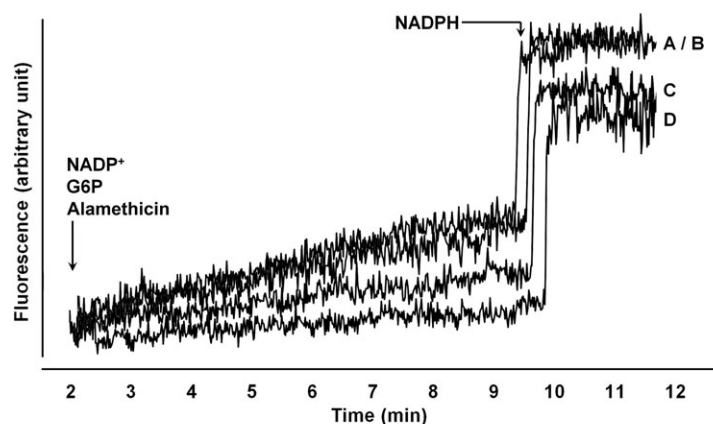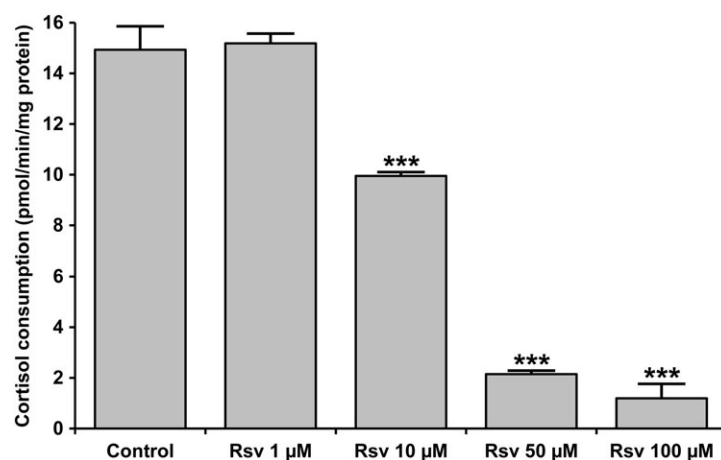

FIG 5 *11β-Hydroxysteroid dehydrogenase type 1 activity. Intact microsomes (1 mg/mL protein) were incubated in a fluorimetric cuvette in MOPS-KCl buffer at 37°C in the absence (trace A) or presence of 10 μM (trace B), 50 μM (trace C), or 100 μM resveratrol (trace D), and the fluorescence of NADPH was recorded continuously. After addition of the substrates (2 mM NADP+ and 100 μM cortisol), 11βHSD1 activity was initiated by permeabilizing the membranes with alamethicin (0.1 mg/mg protein). Detections were finished with the addition of NADPH standard (10 μM). Typical traces of three independent experiments are shown on the top panel, while the calculated enzyme activities are demonstrated as mean $\pm$ SD, n = 3, ***P < 0.0001 versus control on the bottom panel.*

4. Discussion

Obesity is a growing medical problem: the worldwide prevalence nearly tripled between 1975 and 2016 (in 2016, more than 1.9 billion adults were overweight, 650 million of whom were clinically obese) [22]. Obesity is an established risk factor for type 2 diabetes mellitus and a central component of the metabolic syndrome. Therefore, the increasing prevalence of obesity is being paralleled by similar increases in the number of persons with the above diseases [23]. Hence, studies on antiobesity and antidiabetic agents in basic and applied research have received a growing attention in the past decades. The epidemic is still growing not only in developed, but also in developing countries, so new strategies are called for.

Results of both *in vitro* and *in vivo* studies show that the complex metabolism of the major stress hormone cortisol plays

an important role in the pathomechanism of obesity-related metabolic disorders [7]. Influencing the involved enzyme activities is, therefore, considered as a novel strategy for the prevention or treatment of these diseases [24]. As mentioned before, precisely regulated levels of cortisol are essential for adapting to stress, including prolonged starvation. Besides its synthesis and secretion controlled by the hypothalamic–pituitary–adrenal axis, the activity of the hormone is also regulated at a pre-receptor level by 11β-hydroxysteroid dehydrogenase enzymes, which interconvert the active hormone cortisol and its inactive prohormone cortisone [25] i.e., they enhance or diminish the local hormone effects. The enzyme has two isoforms of distinct physiological functions in humans, which differ from each other in tissue specificity, intracellular localization and cofactor demand: type 1 (11βHSD1) and type 2 (11βHSD2) [26,27]. 11βHSD2, a cytoplasmic NAD-dependent enzyme inactivates cortisol in mineralocorticoid target tissues (kidney, colon and salivary glands) to protect the non-selective aldosterone receptor from cortisol excess [28]. In contrast, 11βHSD1, which is an NADP(H)-dependent enzyme localized in the lumen of the endoplasmic reticulum (ER) in glucocorticoid target tissues (liver, skeletal muscle and adipose tissue), modulates glucocorticoid receptor activation by interconverting cortisol and cortisone predominantly toward the production of the active hormone [3,4]. This tissue-specific local cortisol metabolism is largely responsible for the peripheral cortisol effects without remarkably altering the serum hormone levels: the amount of locally produced hormone can reach 25% of that of the newly synthetized cortisol. The liver is responsible for roughly one third and visceral adipose tissue for nearly two thirds of this pre-receptor cortisol production [29]. This system is fueled by glucose 6-phosphate or fructose 6-phosphate from the cytosol [30], and it provides a means of nutrient sensing by linking hexose 6-phosphate supply to glucocorticoid activity [31]. It has been demonstrated that hepatic ER luminal NADPH level and microsomal cortisone reducing capacity are largely determined by the nutritional status (starvation vs. fed state) in rats [21], which further supports the putative role of local cortisol overproduction in obesity-related conditions.

As the repression of peripheral cortisol effect is beneficial in obesity and insulin resistance, research has focused on specific inhibition of 11βHSD1, the isoform that predominantly produces the active hormone. Overexpression of the enzyme in adipose tissue leads to metabolic syndrome in mice [32], while hepatic overexpression causes similar symptoms without obesity [8]. In contrast, 11βHSD1 knockout mice display fasting hypoglycemia, improved lipid and lipoprotein profile, insulin sensitivity, and glucose tolerance [10,11]. They were also found to resist hyperglycemia, due to attenuated gluconeogenic responses [9,11]. Knocking out the enzyme H6PD in mice leads to similar metabolic effects [9,33].

Natural polyphenols, such as EGCG and resveratrol are well-known anti-obesity and anti-diabetic agents; however, the molecular mechanisms leading to these effects are not yet fully explained. We investigated the effect of EGCG on pre-receptor cortisol production earlier, and we found an intriguing mechanism of action. Production of cortisol was remarkably inhibited by the tea flavanol, while the individual activities of the protein components of the luminal cortisone reducing machinery (G6PT-H6PD-11βHSD1) were unaffected. It turned out that EGCG treatment causes the oxidation of luminal NADPH to $NADP^+$ via an enzymatic reaction, yet the enzymes involved remain to be identified. This redox shift in the intravesicular $NADPH:NADP^+$ pool leads to an indirect inhibition of 11βHSD1 mediated cortisol production at a substrate level [12].

The phenomenon called "French paradox" was first published as a result of an epidemiologic study in 1992 [34], and since then it has got a wide scientific publicity. Briefly, it refers to the surprisingly low mortality rate from coronary heart disease (CHD) in France despite elevated risk factors, such as high serum cholesterol level, blood pressure, body-mass index and rate of smoking [35]. The most widely accepted explanation for the "French paradox" is the regular consumption of the polyphenol resveratrol. Inhibitory effect of resveratrol on 11βHSD1 in microsomes prepared from 3 T3-L1-derived adipocytes, rat mesenteric and hepatic tissue has been reported earlier but the exact molecular target remained to be elucidated [36]. The aim of the present study was to reveal if resveratrol shares the mechanism of action with EGCG and whether it also interferes with the cofactor regeneration in the cortisol producing machinery of the ER.

Administration of resveratrol caused a massive inhibition of cortisol production in liver microsomes in a concentration-dependent manner, which was comparable with the effect of EGCG. However, unlike EGCG, resveratrol did not cause a significant redox shift in the luminal $NADPH-NADP^+$ pool, that is, the NADPH level, which drops upon EGCG treatment, was not affected by the addition of resveratrol. The hindered G6P-driven cortisol production in case of resveratrol was not due to a competition for the cofactor but to a direct enzyme inhibition. 11βHSD1 enzyme activity was measured in two ways. The one based on fluorescent detection of NADPH was carried out using high $NADP^+$ concentration for technical reasons, while the one employing HPLC detection of cortisone and cortisol allowed to apply physiological cofactor levels. Data obtained using either method demonstrated that resveratrol acts as a potent inhibitor of 11βHSD1.

Since several molecular targets of resveratrol were identified, the substance retrieved attention as a possible agent against numerous diseases. Clinical significance was examined in a number of studies against type 2 diabetes mellitus, obesity, skin disorders, different types of cancer, neurodegenerative, or cardiovascular diseases. However, no general conclusion can be drawn about the safety range and therapeutic effectiveness, as the consequences are sometimes contradictory, data on capsule versus food form consumption is conflicting and there are uncertain effects of long term dosing. [14,37,38] Our results show a novel molecular mechanism, however, inhibition of the cortisone reducing machinery of the ER is only effective in an early stage of the metabolic syndrome, as the importance of

pre-receptor cortisol activation is diminishing in parallel with the development of the disease. This highlights resveratrol, along with EGCG as an important preventive agent. Unfortunately, there are only a few clinical trials on preventive effects, because long-term treatment and follow-up on numerous healthy individuals were needed, with a given constant dose of resveratrol. As the two mentioned natural polyphenols share the same effect via separate modes of action, a combinational treatment might result in additional efficacy without compromising tolerability.

Regardless of the exact molecular mechanism, repression of prereceptor cortisol production clearly contributes to the beneficial health effects of both EGCG and resveratrol once consumed regularly. Our findings suggest that depletion of microsomal NADPH is not a general polyphenol effect, and they further support that the EGCG-induced NADPH oxidation is due to a specific enzyme activity, which apparently fails to utilize resveratrol.

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