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Attenuation of hepatic expression and secretion of selenoprotein P by metformin

Bodo Speckmann [a,1], Helmut Sies [a,b,c], Holger Steinbrenner [a,*]

[a] Institute for Biochemistry and Molecular Biology I, Heinrich-Heine-University, Düsseldorf, Germany
[b] Institut für umweltmedizinische Forschung (IUF), Heinrich-Heine-University, Düsseldorf, Germany
[c] King Saud University, Riyadh, Saudi Arabia

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ABSTRACT

High serum selenium levels have been associated epidemiologically with increased incidence of type 2 diabetes. The major fraction of total selenium in serum is represented by liver-derived selenoprotein P (SeP). This study was undertaken to test for a hypothesized effect of hyperglycemia and the antihyperglycemic drug metformin on hepatic selenoprotein P biosynthesis. Cultivation of rat hepatocytes in the presence of high glucose concentrations (25 mmol/l) resulted in increased selenoprotein P mRNA expression and secretion. Treatment with metformin dose-dependently downregulated SeP mRNA expression and secretion, and suppressed glucocorticoid-stimulated production of SeP. Moreover, metformin strongly decreased mRNA levels of selenophosphate synthetase 2 (SPS-2), an enzyme essential for selenoprotein biosynthesis. Taken together, these results indicate an influence of metformin on selenium metabolism in hepatocytes. As selenoprotein P is the major transport form of selenium, metformin treatment may thereby diminish selenium supply to extrahepatic tissues.

Introduction

The essential trace element selenium is believed to exert a beneficial influence on human health based on its antioxidative, chemopreventive and antiviral properties [1]. Assumed health benefits prompted consumers in industrialized countries to ingest substantial amounts of dietary supplements, occasionally exceeding adequate selenium intake levels. As the therapeutic window of selenium is narrow, adverse health effects may occur below intake levels required for intoxication [2]. In this regard, the impact of selenium on carbohydrate metabolism and its relevance for type 2 diabetes are subject of an ongoing debate. Insulin-mimetic antidiabetic effects of supranutritional doses of sodium selenate were observed *in vivo* and *in vitro* [3]. In contrast, recent epidemiological studies revealed an association between serum selenium levels and the incidence of type 2 diabetes, pointing to an increased diabetes risk for recipients of long-term high-dose selenium supplementation [4,5].

Around 60% of the selenium circulating in human plasma is present as selenoprotein P (SeP) [6], which has been used as a biomarker for whole body selenium status [7]. Primarily, liver-derived SeP serves as transporter protein, supplying peripheral tissues with selenium [8]. Biological effects of selenium largely rely on selenoenzymes, which contain a selenocysteine residue in their catalytic center. SeP and other selenocompounds stimulate expression and activity of selenoenzymes, most notably cytosolic glutathione peroxidase (GPx-1) [9,10]. GPx-1 detoxifies reactive oxygen species (ROS) such as hydrogen peroxide and organic hydroperoxides, thereby conferring protection against oxidative damage [9–11]. On the other hand, low levels of hydrogen peroxide are required in the early insulin action cascade [12]. Therefore, it is conceivable that high activity of selenoenzymes such as GPx-1 may interfere with insulin signaling. Indeed, transgenic mice overexpressing GPx-1 develop insulin resistance and obesity [13], and increased erythrocyte GPx-1 activity during pregnancy is accompanied by mild insulin resistance in humans [14].

Hepatic selenoprotein P biosynthesis is controlled by insulin and glucocorticoids [15,16]. As SeP is regulated virtually like a gluconeogenic enzyme by key hormones involved in the maintenance of plasma glucose homeostasis [16,17], we hypothesized a link between selenium and carbohydrate metabolism. The present study reveals elevated SeP expression in rat hepatocytes under

Abbreviations: G6Pase, glucose-6-phosphatase; GPx-1, cytosolic glutathione peroxidase; HPRT1, hypoxanthine phosphoribosyl transferase 1; Pstk, phosphoseryl-tRNA [Ser]Sec kinase; ROS, reactive oxygen species; SecS, selenocysteine-tRNA [Ser]Sec synthase; SeP, selenoprotein P; SPS-2, selenophosphate synthetase 2.

* Corresponding author. Address: Heinrich-Heine-University Düsseldorf, Institute for Biochemistry and Molecular Biology I, Universitätsstrasse 1, Geb. 22.03, D-40225 Düsseldorf, Germany. Fax: +49 211 8113029.
E-mail address: Holger.Steinbrenner@uni-duesseldorf.de (H. Steinbrenner).

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hyperglycemic conditions. The antihyperglycemic drug metformin attenuated hepatic expression and secretion of SeP, concomitant with its inhibiting effect on gluconeogenesis. Metformin (1,1-dimethylbiguanide) is one of the most prescribed drugs for treatment of type 2 diabetes [18], as it ameliorates hyperglycemia through suppression of hepatic glucose production [19] and improvement of peripheral insulin sensitivity [20]. Our data suggest that metformin treatment may influence selenium homeostasis of type 2 diabetes patients in addition to its beneficial effects on carbohydrate metabolism.

Materials and methods

Antibodies and reagents. For the antibody against rat SeP, polyclonal antiserum was raised in rabbits immunized with a KLH-linked peptide (H_2N-RGQHRQGHLESDTTAS-$CONH_2$) and affinity-purified by Eurogentec (Seraing, Belgium) as described [16]. The secondary HRP-coupled anti-rabbit IgG antibody was obtained from Dianova (Hamburg, Germany). Metformin, dexamethasone and insulin were from Sigma (Taufkirchen, Germany). Reagents for SDS–PAGE were from Roth (Karlsruhe, Germany). PCR primers were synthesized by Invitrogen (Karlsruhe, Germany).

Isolation and culture of rat hepatocytes. Isolated rat hepatocytes were kindly provided by Dr. R. Reinehr (Clinic for Gastroenterology, Hepatology and Infectiology; Heinrich-Heine-University, Düsseldorf). Hepatocytes were prepared from male Wistar rats as described [21], and grown in William's medium E (Invitrogen) supplemented with 10% fetal calf serum (PAA; Pasching, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (PAA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and serum-starved prior to experimental treatments. As William's medium E does not contain selenium, sodium selenite (200 nM) was added. For the culture of hepatocytes under hyperglycemic conditions, William's medium E containing 11 mmol/l glucose was supplemented with glucose to a final concentration of 25 mmol/l. Non-cytotoxic concentrations of metformin were determined by measuring the ability of the cells to metabolize MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide).

Isolation of RNA and real-time RT-PCR. Total RNA was prepared using the RNeasy Mini Kit (Qiagen; Hilden, Germany). From each sample, 1 μg of RNA was transcribed into cDNA with SuperScript II reverse transcriptase (Invitrogen) and $p(dT)_{15}$ primers (Roche; Mannheim, Germany). Expression of mRNA was analyzed by real-time RT-PCR using the LightCycler system (Roche). PCR was performed with 40 ng cDNA in glass capillaries containing LightCycler FastStart DNA Master SYBR Green I Reaction Mix (Roche), 3 mM $MgCl_2$ and 1 μM of specific primers [16]. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as internal normalization control. Primers were designed using the Universal ProbeLibrary Assay Design Center (Roche) and are listed in Table 1. Specificity of primers was confirmed by melting curve analysis and agarose gel electrophoresis of PCR products.

Immunoblotting. Equal aliquots of culture supernatants of hepatocytes were run on SDS–polyacrylamide gels, and proteins were electroblotted onto nitrocellulose membranes (GE Amersham; Freiburg, Germany). Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 and 5% non-fat dry milk. Immunodetection was carried out using a chemiluminescence-based system (SuperSignal West Pico Substrate; Pierce, Rockford, IL) on Hyperfilm ECL (GE Amersham).

Statistical analysis. Means were calculated from at least three independent experiments, and error bars represent standard deviations (SD). Analysis of statistical significance was done by Student's *t*-test with $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as levels of significance.

Table 1
Sequences of primers for RT-PCR.

Genes	Primers (5′–3′)
SeP	Forward: GCACAGTGACAGTGGTTGCT Reverse: GCTTACTGCTCCCAAGATGC
G6Pase	Forward: TGGAGACTGGTTCAACCTCG Reverse: ACGGTCGCACTCTTGCAGAA
HPRT1	Forward: GACCGGTTCTGTCATGTCG Reverse: ACCTGGTTCATCATCACTAATCAC
SPS-2	Forward: CTTCCGATCGGTTTCTCTTG Reverse: ATTGCCACGGTGGGTAAAG
SecS	Forward: AGACCTGAAAGCCGTGGAG Reverse: AGGCACAGGATATGCTCAGG
Pstk	Forward: GCCTCGTAATTCAGAGTTCAGC Reverse: TGGGATTTTCCAAAGCAGTAA

Results and discussion

Enhanced hepatic selenoprotein P production under hyperglycemic conditions is attenuated by metformin

A plasma concentration of 9 mM glucose is considered as normoglycemic in rats [22]. Diabetic rats exhibit chronically elevated levels of 23 mM glucose and a marked increase in hepatic expres-

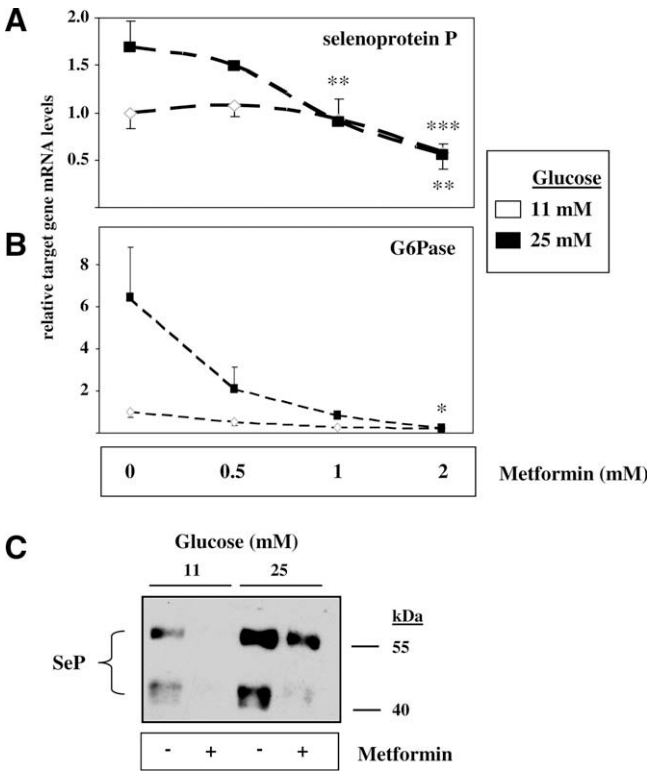

Fig. 1. Attenuation of selenoprotein P mRNA expression and secretion in cultured rat hepatocytes by metformin. Hepatocytes were serum-starved in medium containing 11 or 25 mM glucose and 200 nM sodium selenite for 18 h. Then, cells were incubated in serum-free medium with metformin at the indicated concentrations for additional 24 h. The mRNA levels of SeP (A) and the positive control G6Pase (B) were determined by real-time RT-PCR and normalized against mRNA levels of the housekeeping gene HPRT1. Data are given as means of at least three independent experiments ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control. Secreted SeP was detected by immunoblotting of culture supernatants of rat hepatocytes treated with or without 2 mM metformin for 24 h (C). A representative blot out of four independent experiments is shown.

sion of the gluconeogenic enzyme glucose-6-phosphatase (G6Pase) [22]. We used real-time RT-PCR to test for a hypothesized influence of hyperglycemia on selenoprotein P expression. Rat hepatocytes were cultured for 42 h in William's medium E containing 11 mM or 25 mM glucose. As fetal calf serum (FCS) has been reported to decrease the activity of the selenoprotein P promoter [23], the cells were cultured in serum-free medium in order to exclude interfering effects of serum components. To minimize the impact of accumulation of lactate, the medium was changed after 18 h. Upon cultivation of hepatocytes in hyperglycemic medium (25 mM glucose), SeP mRNA levels were enhanced to 170% ($P < 0.001$) in comparison to cells cultured in the presence of 11 mM glucose (Fig. 1A). This finding adds selenoprotein P to a group of genes upregulated at elevated plasma glucose levels, which are mainly involved in hepatic glucose and lipid synthesis [24]. Enhanced hepatic SeP production under hyperglycemic conditions is consistent with the association between high serum selenium levels and increased incidence of type 2 diabetes found among US-Americans [4]. There is only one selenoprotein besides SeP, the expression of which is known to be glucose-responsive: selenoprotein S (SelS) with a proposed function in maintenance of the cellular redox balance [25]. However, hepatic SelS expression is increased by deprivation of glucose [25], whereas SeP expression is increased under hyperglycemic conditions.

The liver is a major target of the oral antihyperglycemic drug metformin [18], which is taken up by hepatocytes via the organic cation transporter 1 (Oct1) [26]. The antihyperglycemic effect of metformin is partially due to transcriptional repression of gluconeogenic enzymes [19]. We determined whether metformin affects hepatic selenoprotein P mRNA expression as well. The applied doses of metformin ranged from 0.5 to 2 mM, and did not impair hepatocyte metabolic activity as tested by MTT assay (data not shown). Whereas therapeutic concentrations of metformin are rather low with steady-state plasma levels in humans around 10 μM [27], metformin concentrations higher than 180 μM have been measured in the liver of rodents upon oral administration [28]. Pharmacological doses as applied in this study are typically used in experiments exploring metabolic actions of metformin in cultured cells [20,29]. Cellular ATP levels are not affected in rat hepatocytes treated with metformin at doses up to 2 mM [29].

Metformin caused a dose-dependent attenuation of SeP expression: In hepatocytes grown at 11 mM glucose, only the highest dose of metformin (2 mM) had a significant inhibitory effect, decreasing SeP mRNA levels to 50% in comparison to non-treated cells ($P < 0.01$). The downregulation of SeP expression by metformin was more pronounced under hyperglycemic conditions (25 mM glucose), where metformin (2 mM) decreased SeP mRNA levels to 30% of basal values ($P < 0.001$) (Fig. 1A). Recently, we identified the peroxisomal proliferator activated receptor-γ coactivator 1α (PGC-1α) as key regulator of hepatic selenoprotein P biosynthesis [16]. Increased PGC-1α expression has been detected in the liver of diabetic rodents [30]. Consistent with those findings, we measured elevated PGC-1α expression in hepatocytes grown under hyperglycemic conditions, which was counteracted by treatment with 2 mM metformin (data not shown). In parallel with SeP and as positive control [23], we analyzed the mRNA levels of G6Pase. In hepatocytes cultured at 25 mM glucose, G6Pase mRNA levels were strongly increased to 600%, compared to cells grown at 11 mM glucose. Metformin caused a substantial downregulation of G6Pase expression, decreasing G6Pase mRNA levels down to 5% of basal values ($P < 0.05$) (Fig. 1B).

The upregulated SeP mRNA expression in hepatocytes cultured at 25 mM glucose was accompanied by increased SeP secretion, as seen from immunoblots of cell supernatants. Metformin attenuated the secretion of selenoprotein P (Fig. 1C). Rat hepatocytes secrete SeP as two isoforms with different molecular weight [16],

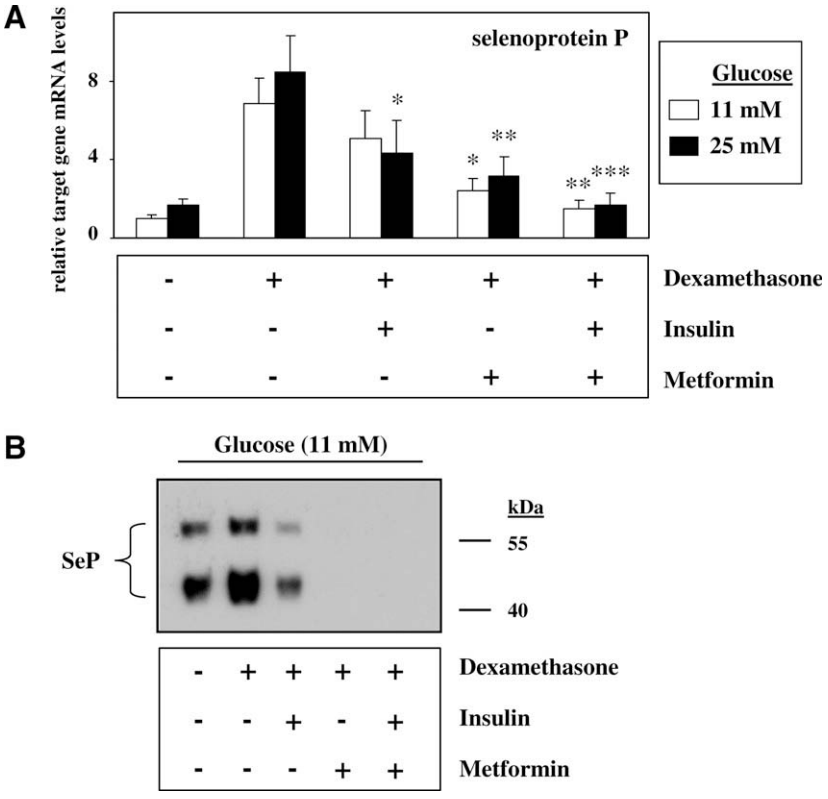

Fig. 2. Metformin counteracts dexamethasone-stimulated expression and secretion of SeP. Hepatocytes were incubated for 24 h in serum-free medium containing dexamethasone (10 μM), insulin (100 nM) and/or metformin (2 mM) as indicated. mRNA levels (A) and secretion (B) of SeP were analyzed as in Fig. 1. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. dexamethasone.

representing a full-length and a truncated form, found in rat plasma as well [31].

Metformin counteracts glucocorticoid-stimulated selenoprotein P production

Administration of glucocorticoids may cause hyperglycemia [32], and metformin has been reported to ameliorate glucocorticoid-induced disturbances of glucose metabolism [33]. Hepatic gluconeogenesis is stimulated by the synthetic glucocorticoid dexamethasone [30]. Recently, we demonstrated elevated selenoprotein P biosynthesis in rat hepatocytes upon treatment with dexamethasone, which was counteracted by insulin [16]. As shown in Fig. 2, metformin counteracted the dexamethasone-induced upregulation of SeP mRNA expression even more efficiently than insulin. Application of metformin and insulin together in the presence of dexamethasone lowered SeP mRNA levels to almost basal values (Fig. 2A).

Dexamethasone-stimulated secretion of selenoprotein P was suppressed by metformin treatment as well, even to a greater extent than SeP mRNA expression. Metformin suppressed the SeP secretion more efficiently than insulin (Fig. 2B). Thus, the inhibitory action of metformin on hepatic gluconeogenesis appears to be paralleled with its suppression of selenoprotein P biosynthesis.

Metformin downregulates mRNA expression of components of the selenoprotein biosynthesis machinery

Concerted downregulation of several enzymes essential for selenium metabolism contributes to the decrease of hepatic selenoprotein P biosynthesis observed during an acute phase response [34]. We hypothesized that metformin might impair the selenoprotein biosynthesis machinery as well, regarding its strong inhibitory effect on SeP secretion (Figs. 1C and 2B). Therefore, the impact of metformin on expression of three key enzymes in selenocysteine biosynthesis was examined: selenophosphate synthetase 2 (SPS-2), selenocysteine-tRNA [Ser]Sec synthase (SecS) and phosphoseryl-tRNA [Ser]Sec kinase (Pstk). As rat SecS and rat Pstk are not yet characterized, we used the rat homologs of SecS (GenBank Accession No. BC166584) and Pstk (GenBank Accession No. XM_001053680), corresponding to the respective mouse and human sequences. The mRNA levels of all three factors were slightly enhanced in hepatocytes cultured at 25 mM glucose. Metformin downregulated the mRNA expression of SPS-2 and SecS. In contrast, Pstk mRNA levels remained unchanged upon metformin treatment. The most pronounced effect was observed on SPS-2 mRNA levels, downregulated by metformin to 40% of basal values ($P < 0.001$) (Fig. 3A).

On the other hand, SPS-2 mRNA expression in rat hepatocytes was enhanced by dexamethasone to 180% of basal values, and metformin suppressed the stimulatory effect of glucocorticoid treatment. In contrast, insulin was not capable of counteracting the dexamethasone-induced stimulation of SPS-2 expression. SecS mRNA levels were influenced neither by dexamethasone nor by insulin, but metformin inhibited the SecS mRNA expression in dexamethasone-treated hepatocytes as well (Fig. 3B).

Thus, the action of metformin is not limited to downregulation of selenoprotein P expression but extends to components of the selenoprotein biosynthesis machinery. The strongest inhibitory effect of metformin was observed regarding SPS-2, the key enzyme

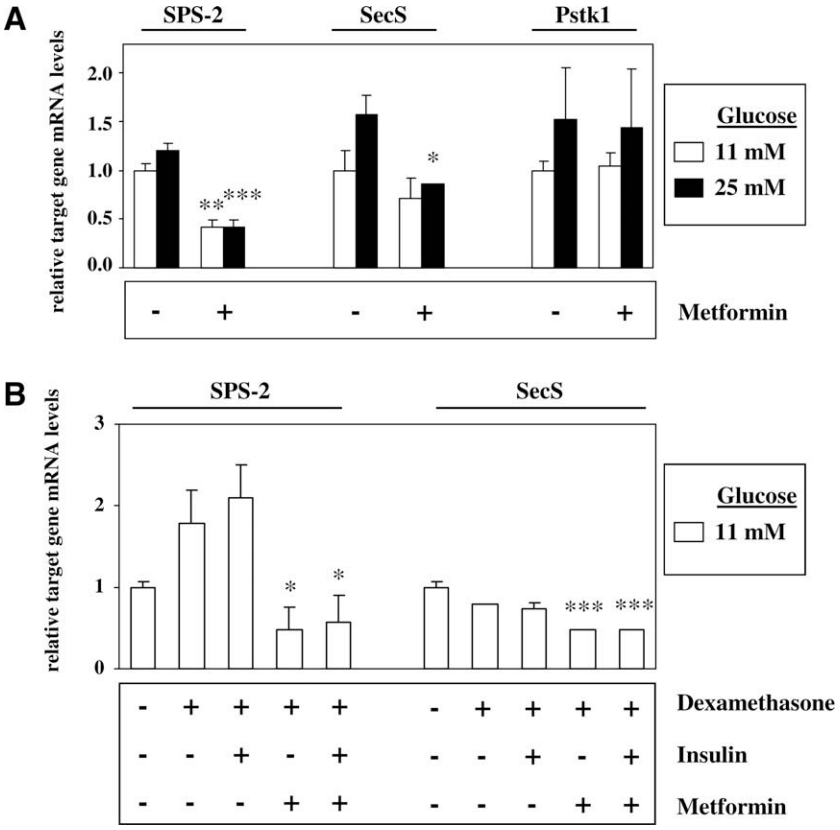

Fig. 3. Downregulation of selenoprotein biosynthesis factors by metformin. Hepatocytes were incubated for 24 h with or without 2 mM metformin. The mRNA levels of genes encoding selenophosphate synthetase-2 (SPS-2), selenocysteine synthetase (SecS) and phosphoseryl-tRNA[Ser]Sec kinase (Pstk) were determined by real-time RT-PCR and normalized against HPRT1. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control. (A). Hepatocytes were treated for 24 h with dexamethasone (10 μM), insulin (100 nM) and metformin (2 mM) as indicated. The mRNA levels of SPS-2 and SecS were determined and normalized against HPRT1. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. dexamethasone. (B). Data represent means of three independent experiments ± SD.

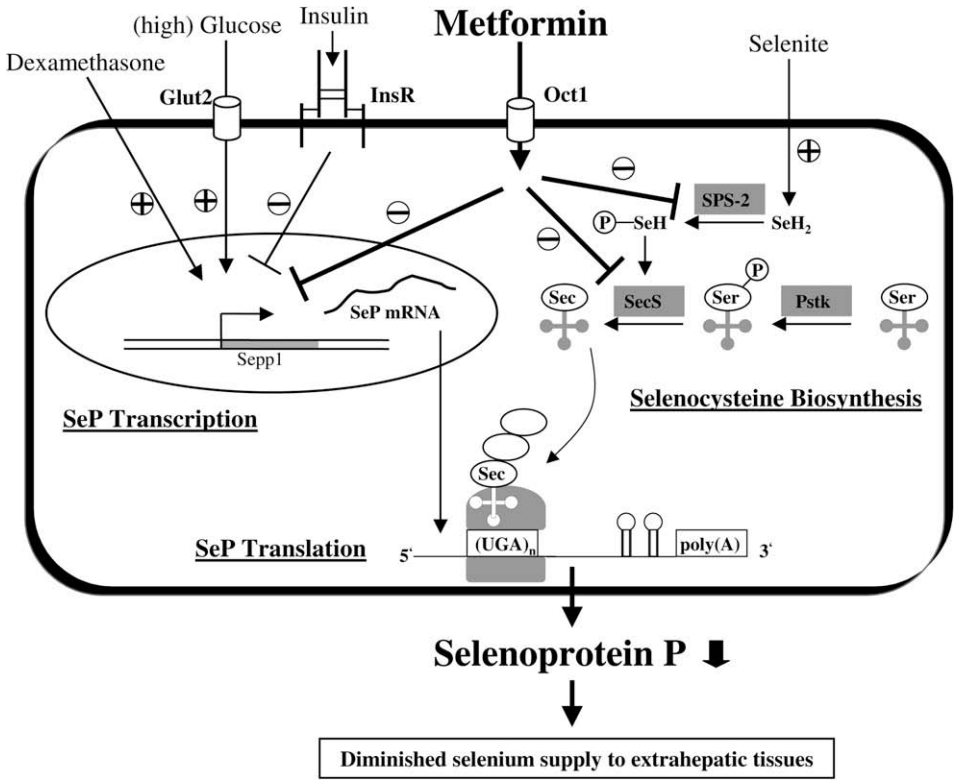

Fig. 4. Regulation of hepatic SeP biosynthesis by metformin. Metformin attenuates SeP transcription, stimulated by dexamethasone or high glucose concentrations. Additionally, metformin downregulates expression of essential enzymes for selenocysteine biosynthesis (SPS-2, SecS), resulting in lowered hepatic SeP secretion and (presumably) diminished selenium supply of extrahepatic tissues. See Conclusions for further details.

for generation of the active selenium donor for selenocysteine biosynthesis in mammals [35]. During all experiments, the hepatocytes were well supplied with selenium. The used dose of 200 nM sodium selenite is sufficient to ensure optimal hepatic biosynthesis of selenoprotein P [1].

Conclusions

This study expands the range of metabolic actions of the antidiabetic drug metformin to selenoproteins. As depicted in Fig. 4, metformin downregulates the hepatic mRNA expression of selenoprotein P along with two enzymes required for incorporation of selenocysteine into selenoproteins, which altogether results in decreased selenoprotein P secretion. Moreover, metformin suppresses the elevation of SeP biosynthesis caused by high glucose concentrations or dexamethasone. As selenoprotein P represents the major physiological transport form of selenium, metformin may thereby impair expression and activity of selenoenzymes in extrahepatic tissues by diminishing their selenium supply. This idea is supported by a study of Pavlović et al.: a two-week metformin treatment resulted in decreased activity of the selenoenzyme GPx-1 in erythrocytes of obese patients with type 2 diabetes [36].

Currently, there is a debate on the safety of selenium supplementation with respect to type 2 diabetes. As selenium is appreciated for its antioxidant capacity due to incorporation into ROS-detoxifying selenoenzymes [1], an antidiabetic effect of selenium supplementation would be expected. However, recent studies suggest in contrast that long-term consumption of selenium supplements disturbs the carbohydrate metabolism and increases the risk for obesity and type 2 diabetes in both humans and animals [4,5,37]. Regarding the role of oxidative stress as a key player in development of late diabetic complications [38], these reports appear paradoxically. But the paradox may be resolved by the observation that low levels of hydrogen peroxide enhance insulin signaling through inhibition of the insulin antagonistic protein tyrosine phosphatase 1B [12], which is itself activated by selenium [37]. Thus, a metformin-induced attenuation of selenoprotein P biosynthesis might contribute to the well-known improvement of peripheral insulin sensitivity elicited by this antidiabetic drug [20] via decreasing selenium bioavailability in extrahepatic tissues.

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