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Effect of PAO (phenylarsine oxide) on the Inhibitory Effect of Insulin and IGF-1 on Insulin Release from INS-1 Cells

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Abstract. Phenylarsine oxide (PAO) which complexes vicinal thiol groups is a valuable pharmacological tool to investigate the interaction of peptides such as insulin with their receptors and the signal transduction from the receptor to the cell interior. This tool was now used to elucidate the inhibitory effects of insulin and IGF-1 on insulin secretion via their receptors. Insulin and IGF-1 inhibited insulin release from INS-1 cells, an insulin secreting cell line. PAO was able to reverse this inhibitory effect of both hormones. Dimercaptopropanol (DMP), which is well known to antagonize PAO effects, inhibited the abolishment of PAO effect on the inhibitory effect of insulin and IGF-1 regarding insulin release. Membrane bound GLUT2 in INS-1 cells was increased by either insulin and IGF-1 which is counteracted by PAO. Thus the inhibitory effect of insulin and IGF-1 on insulin release is operative and can be disturbed by a thiol interacting compound such as PAO. This may happen at the receptor level or at the sub-receptor level.

Key words: INS-1 cells, Inhibition of insulin release, GLUT2, Phenylarsine oxide, Dimercaptopropanol

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PHENYLARSINE oxide (PAO), a trivalent arsenical and mono-substituted organoarsenical is able to complex vicinal thiols; many SH-dependent processes, therefore, can be affected. It acts by forming cyclic thioarsenites with adjacent SH groups [1] and is a biochemical tool for probing and blocking sulfhydryl-dependent processes. PAO is also a tyrosine phosphatase inhibitor thus increasing the phosphorylation status of various peptides. Taken together PAO is a valuable tool in various aspects which are summarized below [1–5]:

With respect to insulin receptors there is an

(a) Inhibition of down-regulation of insulin receptors
(b) Inhibition of protein internalisation including insulin endocytosis
(c) Regulation of insulin receptor autophosphorylation under defined conditions (not unequivocal!) [6–8] *e.g.* in the presence of vanadate; PAO thereby probably bypasses the direct effects on the insulin receptor and will interact with the components of insulin signal transduction [9, 10].

With respect to biological effects there is an

(a) Inhibition of insulin effect on various parameters and enzymes (fructose 2,6-biphosphate, phosphofructokinase, glucokinase, glycolysis, glycogen synthesis, amino acid transport, glycogenolysis, proteolysis)
(b) Inhibition of glucose transport even under conditions when insulin receptor binding/autophosphorylation is not influenced [11–13]; inhibition of IGF-1 — induced glucose transport
(c) Decrease in GLUT4 [14], albeit no down-regulation of the GLUT1 transporter and inhibition of the insulin-induced dephosphorylation of GLUT4
(d) Increase in amino acid uptake [15]
(e) Inhibition of the insulin-induced PP-1 activation and inhibition of phosphotyrosine phosphatase (GAP-phosphorylation on tyrosine (p21 ras-GTPase activation protein), increased GAP-association with the insulin receptor, inhibition of insulin-induced p21 ras activation) [16]
(f) inhibition of PI3-kinase a which is recruited from IRS-1 (insulin receptor substrate 1) tyrosine phosphorylation [17].

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The latter is compatible with the view that PAO enters the cell [12]; thus its effect is not only mediated by interaction with membrane proteins or receptors.

PAO effects are at least partly reversed by DMP (dimercaptopropanol = BAL, British antilewisit) as well as DTT (dithiothreitol): an example is the inhibition of PAO-influenced transport processes and the PAO-induced phosphorylation of pp15. PAO effects can be antagonized by dithiols such as DMP but not monothiols. DMP, therefore, is a valuable tool.

Insulin secretion was shown to be under negative feedback control: Various approaches are in favour of this hypothesis such as a direct inhibitory effect of insulin on insulin release was observed [18, 19], an increase in insulin release was indirectly mediated by the presence of insulin antibodies [20], and an increase was also observed by modifying the perfusion rate of the endocrine pancreas *in vitro* [21]. Since insulin effects are generally affected by PAO as was mentioned before, PAO effects on insulin-modulated insulin release were investigated using INS-1 cells, an insulin secreting cell line. Since there is an interaction between PAO and IGF-1 and since IGF-1 receptors may be involved in insulin negative feedback effects on insulin release [22, 23], the interaction between IGF-1 and PAO was investigated as well.

Methods

Chemicals

Crystalline pork insulin (26 U/mg) was provided by Aventis, FRG. IGF-1, PAO (phenylarsine oxide) and DMP (2,3-dimercapto-1-propanol = BAL) were from Sigma, Germany: a 10 mM stock solution of PAO was prepared by dissolving 17 mg in 1 ml DMSO and adding 9 ml H_2O. The final concentration of DMSO (ranging up to 0.004% did not affect insulin release (data not shown).

Insulin antibodies were from Linco (USA), supplied by Biotrend GmbH (Germany). Anti-GLUT2 antibodies were obtained from Biogeneses (Dunn Labortechnik GmbH, Germany).

INS-1 cell culture and incubation

INS1 cells [24] generously provided by Dr. C. Wollheim (Geneva, Switzerland) were plated at a density of 5×10^5 cells/ml and grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 50 μM mercaptoethanol, 100 U/mL penicillin and 0.1 mg/mL streptomycin.

In the literature PAO was used at a maximum concentration of 5 μM and DMP at 200 μM. PAO is difficult to handle since it inhibits "inhibitory" phosphatases at low and induces "stimulatory" phosphatases at higher concentrations [25] and is also toxic at higher concentrations. Cells were checked for signs of toxicity (release of LDH = lactate dehydrogenase). These signs were observed at concentrations of 5 μM, but not at lower concentrations (data not shown) which were used in our experiments.

Insulin content

INS-1 cells were washed twice with Krebs-Ringer buffer (KRB). Extraction of insulin was performed according to the acid ethanol technique of Scott and Fisher [26], employing 1 ml of solution for each well. The cells were sonicated and extracted overnight at 4°C. Aliquots were taken and stored at –20°C until determination of insulin by RIA.

LDH

Release of LDH was determined according to the method of Trautschold *et al.* [27] adapted from Bergmeyer [28]. NAD was determined fluometrically (pyruvate + NADH → lactate + NAD).

Insulin secretion and radioimmunoassay

INS-1 cells were plated at 1.5×10^5 cells/well in 24-well cell culture plates and cultured for 5 days. Cells were washed three times with Krebs-Ringer buffer containing 10 mM HEPES and 0.5% bovine serum albumin (KRH-buffer). The cells were incubated with KRH-buffer containing 8.3 mM glucose in the presence or absence of the respective compounds for 90 minutes as indicated in the legends to the figures. Insulin release into the medium was determined by a radioimmunoassay using rat insulin as a standard, (mono-^{125}I-Tyr^{A14})-porcine insulin as the labeled compound and anti-insulin antibodies. The antibodies cross-react with porcine and rat insulin. The intraassay and interassay variabilities were 4.2 and 9.8%, re-

spectively. Each test compound has been checked for non-interference with the insulin radioimmunoassay.

Western blotting for GLUT2

INS-1 cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) by centrifugation at 1000 g for 10 min at 4°C. The cell pellets were resuspended in 2 volumens of lysis buffer (5% (w/v) SDS, 80 mM Tris, pH 6.8, 5 mM EDTA, 0.5 mM PMSF (phenylmethylsulfonyl fluoride) at 4°C, incubated on ice for 15 min and sonicated (three times for 10 s). 2 M KCl were added to give a final concentration of 0.4 M followed by centrifugation at 100,000 g for 60 min at 4°C. The protein yield was estimated by the bicinchoninic acid (BCA)-assay. Lysates were added to 2 × SDS PAGE buffer (Laemmli), subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked overnight at 4°C in blocking buffer (5% (w/v) BSA, 0,05% (w/v) Tween 20 in PBS buffer). Primary antibodies (Anti-GLUT2) were incubated with membranes for 1 h at room temperature. Biotinylated mouse anti-rabbit secondary antibodies and subsequent streptavidin-peroxidase conjugate were incubated with membranes likewise for 1 h at room temperature. Washes and chemiluminescence detection were performed by the enhanced chemiluminescence (ECL)-systems (Amersham) according to the manufacturers protocol. Quantitative assessment of GLUT2 protein expression was performed by laser densitometric scanning of the band intensities.

Statistics

Results are shown as means ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) (RS/1 statistics pack, BBN Software Products Corp.) followed by a *post-hoc* test (Newman Keuls). A p value<0.05 was considered significant.

Results

1. PAO effect on insulin release (Fig. 1)

PAO inhibited the glucose (8.3 mM)-induced insulin release in a concentration-dependent manner. The maximum PAO concentration used was 3.0 μM which is not toxic (see above). PAO did not affect insulin release at a substimulatory glucose concentration (3.0 mM).

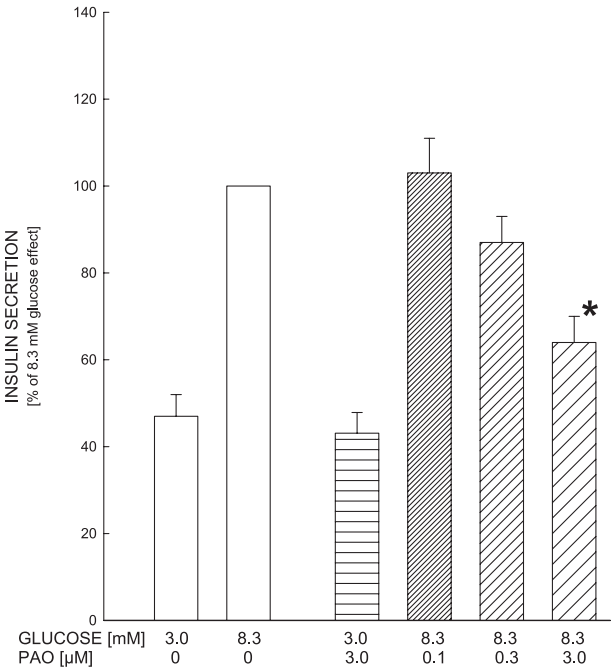


Fig. 1. Effect of glucose (3.0 and 8.3 mM) and of various concentrations of PAO on insulin secretion in the presence of 8.3 mM glucose. INS-1 cells were incubated for 90 min in the presence of the compounds. Insulin was determined by RIA using a rat insulin standard. Mean ± SEM of 6 independent experiments. * P<0.05 vs. glucose (8.3 mM) alone.

The insulin content was 2923 ± 178 μU/10^6 cells. The fractional insulin release was 6.14 ± 0.25% over 90 min. The insulin content varied between 2641 and 3188 μU/10^6 cells; there was no statistically significant change of insulin content or a correlation with respect to the compounds PAO and DMP used. Release data, therefore, were not normalized to the insulin content. Release data were normalized to the effect at a stimulatory (8.3 mM) glucose concentration.

2. Interaction with insulin (Fig. 2)

Both PAO (3 μM) and insulin (1000 μU/ml) decreased glucose (8.3 mM)-induced insulin release. PAO fully reversed the inhibitory effect of insulin. This is interesting since PAO itself inhibited insulin release; if this effect is subtracted, the reversion of the insulin effect is even more impressive.

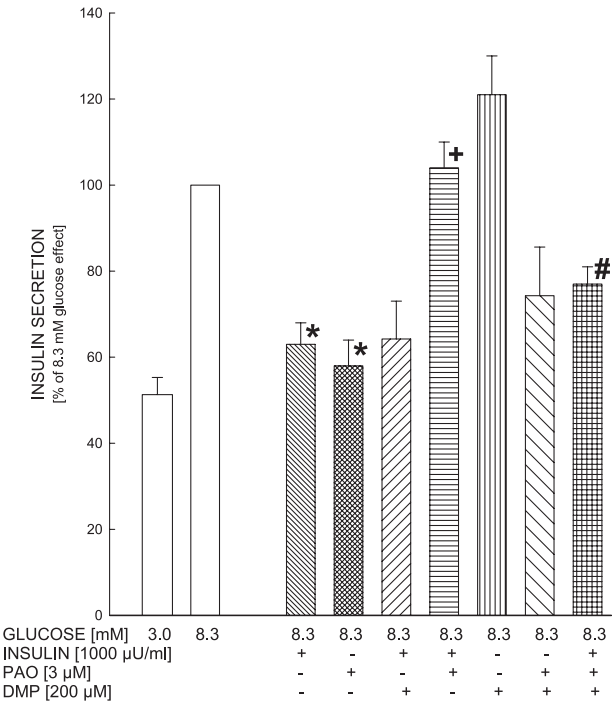


Fig. 2. Effect of glucose (3.0 and 8.3 mM) and of various combinations of insulin, PAO and DMP concentrations on insulin secretion in the presence of 8.3 mM glucose. INS-1 cells were incubated for 90 min in the presence of the compounds. Insulin was determined by RIA using a rat insulin standard. Mean ± SEM of 6 independent experiments. * P<0.05 vs. glucose (8.3 mM) alone; $^{+}$ p<0.05 vs. insulin effect; $^{\#}$ p<0.05 vs. PAO and insulin effect.

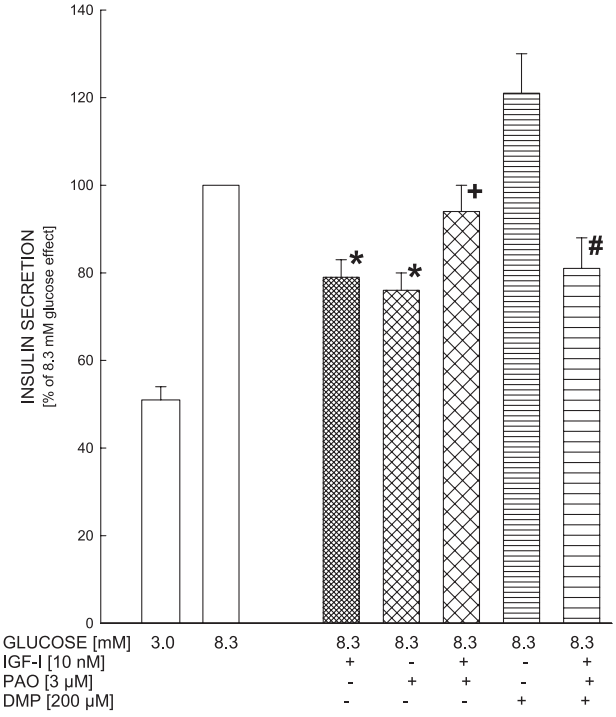


Fig. 3. Effect of glucose (3.0 and 8.3 mM) and of various combinations of IGF-1, PAO and DMP concentrations on insulin secretion in the presence of 8.3 mM glucose. INS-1 cells were incubated for 90 min in the presence of the compounds. Insulin was determined by RIA using a rat insulin standard. Mean ± SEM of 6 independent experiments. * P<0.05 vs. glucose (8. 3 mM) alone; $^{+}$ p<0.05 vs. insulin effect; $^{\#}$ vs. PAO and insulin effect.

DMP (dimercaptopropanol = BAL) alone had a marginal stimulatory effect on insulin release. It partly antagonized the inhibitory effect of PAO on the insulin effect but did not influence the insulin effect *per se*.

3. Interaction with IGF-1 (Fig. 3)

The same type of experiments was performed except that IGF-1 (10 nM) was used instead of insulin. PAO reversed the inhibitory effect of IGF-1. This again is interesting since PAO itself inhibited the insulin release; if this effect is subtracted, the reversion of the IGF-1 effect is even more impressive.

DMP antagonized the inhibitory effect of PAO on the IGF-1 effect.

4. Membrane GLUT2 (Fig. 4)

The membrane bound GLUT2 amount normalized for β-actin protein was increased in membranes of INS-1 cells by either insulin and IGF-1. This effect was counteracted by addition of 3 µM PAO.

Discussion

Insulin and IGF-1 are well known to inhibit insulin release albeit it is not unequivocally accepted (see above). The effect of insulin is most prominent at rather high concentrations being present in the intercellular space of pancreatic B-cells which is in favour of an interaction of insulin with IGF-1 receptors as was postulated by Schravendijk [22, 23]. PAO reversed the inhibition of insulin release in the case of both insulin and IGF-1. This may strengthen the argument that insulin and IGF-1 act via the same receptor as was suggested [22, 23].

DMP is able to at least partly antagonize the inhibi-

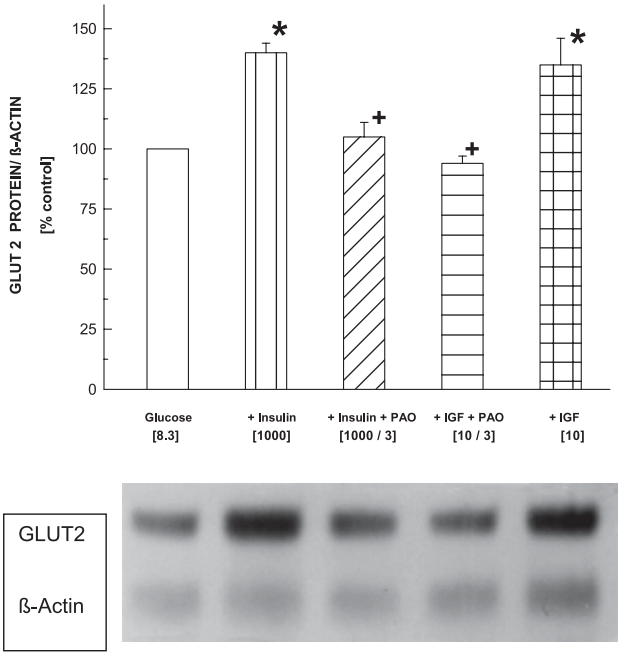


Fig. 4. Effect of insulin (1000 μU/ml) and of IGF-1 (10 nM) in the absence and presence of PAO (3 μM) on GLUT2. Data are corrected for β-actin protein measurement. One representative blot out of 4 independent experiments is shown. Mean ± SE; * $p<0.05$ vs. glucose alone; $^{+}$ $p<0.05$ vs. insulin and IGF-1 effect, respectively.

tory effects of PAO on either the insulin and the IGF-1 effect. This tool helps to interpret the PAO effect as a selective effects. SH groups are involved in the effects of both hormones.

The amount of GLUT2 is increased by both insulin and IGF-1. The interaction of PAO with either hormone effect is shown by abolishing their effects on GLUT2 transporters. With respect to the mechanism in other systems PAO is known to block the turnover of the phosphoryl group transmission from the receptor to the glucose-transport system [29].

The data strengthen the negative feedback of insulin on insulin release and the inhibitory effect of IGF-1; they additionally indicate the involvement of SH groups in this process within the signalling machinery. The biological effect of the hormones on the glucose transporters is influenced in INS-1 cells as well.

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