Inhibition of Insulin-Stimulated Xylose Uptake in Denervated Rat Soleus Muscle: a Post-receptor Effect

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Summary. Insulin (100 U/l) stimulated xylose uptake in rat soleus muscle from a basal value of 2.3 ± 0.5 to 11.6 ± 2.1 $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. Denervation (section of the sciatic nerve) markedly reduced the stimulatory action of insulin (basal 1.3 ± 0.4 $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$; insulin-stimulated 4.5 ± 0.6 $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). This effect appeared 3 days after denervation and was maximal after 5 days. Denervation also affected the insulin dose response curve; there was no effect of insulin in denervated muscle until the concentration exceeded 1 U/l. Denervation inhibited insulin-stimulated α -aminoisobutyrate uptake by 77% but did not affect ^{125}I -insulin binding or glucose-independent activation of glycogen synthase by insulin. There was no effect of denervation on the insulinomimetic ef-

fects of concanavalin A, hydrogen peroxide, vitamin K₅, anoxia, 2,4-dinitrophenol, cooling, hyperosmolarity or EDTA, but the effect of diamide was inhibited. It is concluded [1] that denervation inhibits insulin-stimulated sugar transport at some early post-receptor step, and [2] that the mechanism whereby insulin activates glycogen synthase is different from the activation of the membrane transport of sugars and amino acids.

Key words: Denervation, insulin-resistance, sugar transport, amino acid transport, glycogen synthase, ^{125}I -insulin binding, concanavalin A, diamide.

Considerable progress has been made in recent years concerning the interaction between insulin and its receptor [1, 2]. More recently, it has been suggested that insulin stimulates sugar transport by promoting the translocation of sugar carriers from intracellular sites to the plasma membrane [3, 4]. However, the nature of the signalling mechanism which couples the insulin receptor to the sugar transport system remains obscure. It has been demonstrated that insulin induces the release of a chemical messenger from the plasma membrane which mediates the effect of the hormone on the intracellular enzymes, glycogen synthase and pyruvate dehydrogenase [5–7]. This factor has not yet been associated with insulin-stimulated sugar transport.

The coupling system can be studied using agents which block insulin action at the post-receptor level. Buse and Buse [8] reported that denervation (section of the phrenic nerve) abolished the stimulatory effect of insulin on glucose uptake by rat hemidiaphragm. They found that denervation inhibited insulin-stimulated D-xylose uptake by diaphragm in vitro and in vivo [9]. Denervation did not affect total [^{131}I]insulin binding by diaphragm in vitro and in vivo [8], which suggests that the insulin-resistant state induced by denervation is due

to some post-receptor lesion. The nature of this defect does not appear to have been further studied. The experiments reported in this study were designed to observe the effects of denervation on insulin-stimulated muscle sugar transport in rat soleus muscle.

Methods

Soleus muscles were removed from denervated and control rats (50–60 g). Muscles were incubated at 37 °C (unless otherwise stated) under an atmosphere of O₂-CO₂ (95:5, v/v); anaerobic incubations were under N₂-CO₂ (95:5, v/v). The basic ('bicarbonate') medium contained NaCl (118 mmol/l), KCl (4.8 mmol/l), CaCl₂ (2.6 mmol/l), MgSO₄ (1.2 mmol/l), KH₂PO₄ (1.2 mmol/l) and NaHCO₃ (25 mmol/l); before use the medium was gassed with the appropriate gas mixture. Additions to, or variations from, this basic medium are detailed in the text.

Sugar Transport

Xylose uptake was determined using the method of Korbl et al. [10]. In this procedure the muscles are first preincubated in 0.5 ml of bicarbonate medium under the test conditions; the uptake of D-[U- ^{14}C] xylose (final concentration 10 mmol/l, specific activity 0.03 $\mu\text{Ci}/\mu\text{mol}$) is then measured over a 5 min period, using D-[1- ^3H] sorbitol (10 mmol/l, specific activity 0.1 $\mu\text{Ci}/\mu\text{mol}$) as the extracellular marker.

Fig. 1. Effect of denervation on basal and insulin-stimulated xylose uptake. Soleus muscles were taken from rats before and up to 7 days after denervation. Muscles were incubated first for 30 min at 37 °C in bicarbonate medium and then for 30 min in the presence (●) or absence (○) of insulin (100 U/l). Each point is the mean $\pm$ SEM of five determinations

Fig. 2. Effect of denervation on the insulin dose response curve. Muscles from either normal (○) or 7-day denervated (●) rats were incubated first for 15 min at 37 °C in bicarbonate medium and then for a further 15 min in the presence or absence of insulin (10^{-5} – 10^0 mU/l). The square symbols indicate control experiments where normal (□) and denervated (■) muscles were incubated with deactivated insulin. Each point is the mean $\pm$ SEM of five determinations

¹²⁵I-insulin Binding

Beef insulin was iodinated using carrier-free sodium [¹²⁵I]iodide and chloramine T according to the method of Greenwood et al. [11]. Labelled insulin was separated by chromatography on Sephadex G-50. The purity of each fraction was checked by paper electrophoresis; only those fractions in which at least 95% of total ¹²⁵I was present as ¹²⁵I-insulin were used. ¹²⁵I-insulin binding to soleus muscle was measured using the procedure developed by Yu and Gould [12]. Soleus muscle pairs were incubated for 90 min at 25 °C in 1 ml of bicarbonate medium containing bovine serum albumin (2 mg) and ¹²⁵I-insulin

(0.17 nmol/l, specific activity 25–50 Ci/g, equivalent to 0.07–0.14 atoms ¹²⁵I/mol insulin) $\pm$ unlabelled insulin (up to 3.3 μ mol/l). The muscles were washed twice at 0 °C in 5 ml of bicarbonate buffer, first for 30 min, then for 60 min, then homogenized in 2 ml of trichloroacetic acid (50 g/l). The homogenizer was rinsed twice with 2 ml of trichloroacetic acid (50 g/l); the combined homogenate and rinsings were filtered through a 2.5 cm Whatman GF/A glass fibre disc in a Millipore filter. The precipitate on the filter was washed three times, each with 5 ml of ice-cold trichloroacetic acid (50 g/l), then counted in a Philips PW 4520 gamma counter. Specific ¹²⁵I-insulin binding was calculated as the difference between ¹²⁵I-insulin bound in the presence of tracer alone ('total binding') and in the presence of tracer plus 3.3 μ mol unlabelled insulin/l ('non-specific binding').

Amino Acid Transport

Uptake of α -aminoisobutyrate was assayed by incubating muscles for 15 min in 0.5 ml of bicarbonate buffer containing 1 mmol/l α -[1-¹⁴C] aminoisobutyrate (specific activity 0.05 μ Ci/ μ mol) and 10 mmol/l D-[1-³H]sorbitol (specific activity 0.5 μ Ci/ μ mol) as an extracellular marker. The muscles were dissolved in NCS tissue solubilizer and counted in a similar manner to those used for the determination of xylose uptake.

Glycogen Synthase

Following the appropriate incubation, the muscles were frozen in liquid nitrogen and held at –70 °C. Each muscle was added to 0.2 ml of a solution containing Tris-HCl buffer (10 mmol/l, pH 7.8), EDTA (10 mmol/l), NaF (50 mmol/l) and glycerol (600 ml/l) in a small glass homogenizer (internal diameter 8 mm) pre-frozen in liquid nitrogen. The frozen muscle and buffer were crushed together into a powder using the Teflon pestle manually. To this mixture was added 0.6 ml of a solution containing Tris-HCl buffer (10 mmol/l, pH 7.8), EDTA (5 mmol/l), NaF (50 mmol/l), mercaptoethanol (50 mmol/l) and bovine serum albumin (1 g/l). The contents of the tube were allowed to warm to 0 °C in an ice bath, whereupon they were homogenized for 20–25 s to achieve an even homogenate. This was transferred to a plastic tube and centrifuged in an Eppendorf 5412 microfuge for 5 min. The supernatant solution was applied to a Sephadex G-25 column (Pharmacia, PD-10) equilibrated with Tris-HCl buffer (10 mmol/l, pH 7.8) containing EDTA (5 mmol/l), NaF (50 mmol/l) and mercaptoethanol (50 mmol/l). The column was developed with the same buffer. The elution of the protein peak was monitored using the Coomassie blue reagent described by Bradford [13]; the protein-containing fractions were pooled. Glycogen synthase was assayed using the method of Thomas et al. [14]. The percentage of synthase in the active, I form was determined by assaying the enzyme in the absence (synthase I) and presence (total synthase I + D) of 20 mmol glucose-6-phosphate/l.

Denervation

Sprague-Dawley rats weighing 50–60 g were anaesthetized with ether. After treating the area with a solution of hibitane (5 ml hibitane, 10 ml water, 85 ml absolute ethanol), a small incision was made in the skin on the lateral aspect of each thigh and the sciatic nerve exposed. A section of nerve approximately 5 mm in length was removed and the wound closed with Michel clips. The animals were held for up to 7 days before further experimentation. Muscles taken from rats (50–60 g) at the time of operation weigh 20–25 mg; muscles from normal rats (70–90 g) one week later weigh 30–35 mg. Following denervation, muscles do not increase in mass; 1 week later they still weigh 20–25 mg. Basal xylose uptake in normal 20–25 mg muscles was determined to be $2.6 \pm 0.1 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ($n = 9$) and in normal 30–35 mg muscles taken from litter-mates one week later $3.0 \pm 0.2 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ($n = 9$; NS). Accordingly, control muscles were taken from 50–60 g rats so that their weight would be the same as the denervated muscles.

Fig. 3 A and B. ^{125}I -insulin binding by normal and denervated soleus muscle. **A** Muscles were incubated for 90 min at 25°C in the presence of ^{125}I -insulin (0.17 nmol/l) and various concentrations of unlabelled insulin (up to $3.3\text{ }\mu\text{mol/l}$). **B** Scatchard plots derived from the data shown in **A**. $\circ$ = normal muscle, $\bullet$ = denervated muscle. Each point is the mean of ten determinations

Table 1. Effect of denervation on insulin stimulation of amino acid transport and glycogen synthase in rat soleus muscle

	Dener- vated	Basal	Insulin
α -Aminoisobutyrate uptake	—	0.77 ± 0.24 (5)	1.67 ± 0.12 (5) ^b
($\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)	+	0.84 ± 0.07 (5)	1.05 ± 0.07 (5) ^a
Glycogen synthase	—	15.0 ± 0.7 (4)	20.9 ± 1.9 (4) ^c
(% synthase I)	+	15.1 ± 1.2 (4)	21.6 ± 0.9 (4) ^c

α -aminoisobutyrate uptake: soleus muscle pairs were incubated for 30 min at 37°C in bicarbonate medium $\pm$ insulin (100 U/l); the uptake of α -[^{14}C] aminoisobutyrate was then determined as detailed under Methods. Glycogen synthase: soleus muscle pairs were preincubated for 30 min at 37°C in bicarbonate medium; insulin (100 U/l) was added to one muscle from each pair and the muscles incubated a further 15 min.

Results are expressed as mean $\pm$ SEM with number of determinations in parentheses. Insulin versus basal: ^a = $p < 0.01$, ^b = $p < 0.005$, ^c = $p < 0.001$

Materials

D-[U- ^{14}C]xylose, sodium [^{125}I]iodide and NCS tissue solubilizer were from Amersham Australia Pty., Sydney, Australia. D-[1- ^3H]sorbitol and α -[1- ^{14}C]aminoisobutyrate were from New England Nuclear Corporation, Boston, USA. Beef insulin, twice recrystallized, was from the Commonwealth Serum Laboratories, Melbourne, Australia. A sample of insulin was deactivated by heating a solution containing insulin (4 g/l), urea (6 mol/l) and dithiothreitol (13 mmol/l), pH 3.0, for 30 min at 60°C , then neutralizing to pH 7.0. 2,4-dinitrophenol and EDTA were from British Drug House Chemicals Australia, Sydney. Concanavalin A was from Boehringer-Mannheim (Australia), Sydney. Mannitol was from Calbiochem-Behring (Australia), Sydney. Vi-

tamin K₅ was from Tokyo Kasei Kogyo, Tokyo. α -aminoisobutyrate, diamide, dithiothreitol and rabbit liver glycogen were from Sigma Chemicals, St. Louis, USA.

Statistics

To minimise the effect of biological variation between individual animals, wherever possible the experiments were designed using paired controls. One muscle from each pair was incubated under test conditions, while the second served as the control. The results of these experiments were analyzed for statistical significance using Student's t-test as applied to paired samples. Where it was not possible to use paired controls, muscles taken from litter-mates were distributed randomly among the experimental groups and the results subjected to statistical analysis using the standard Student's t-test.

Results

In normal muscle, insulin (100 U/l) stimulates xylose uptake by 510% (from a basal value of 2.3 ± 0.5 to $11.6 \pm 2.1\text{ }\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ($n = 5$, $p < 0.001$)). Following denervation the stimulatory effect of insulin was markedly reduced (Fig. 1). The effect of denervation was apparent after 3 days and became maximal after 5 days. Denervation did not abolish insulin action completely; in denervated muscle (5 days) insulin stimulated xylose uptake by only 170% (from a basal value of 1.3 ± 0.4 to $4.5 \pm 0.6\text{ }\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, $n = 5$, $p < 0.02$).

The effect of denervation on the insulin dose response curve is shown in Figure 2. In normal muscle the effect of insulin becomes apparent above 10 mU/l and is maximal at 10 U/l . In contrast, there was no effect in

Table 2. Effect of denervation on activators of muscle sugar transport in rat soleus muscle

Activator	Period of exposure to activator (min)		Xylose uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)		Stimulation (%)
			Basal	Activated	
Xylose (30 mmol/l)	0 ^a	normal	2.2 $\pm$ 0.2	7.9 $\pm$ 0.8 ^g	259
		denervated	2.6 $\pm$ 0.5	10.4 $\pm$ 3.2 ^g	300
Concanavalin A (500 mg/l)	30	normal	2.3 $\pm$ 0.4	6.0 $\pm$ 0.2 ^f	161
		denervated	1.9 $\pm$ 0.1	4.5 $\pm$ 0.3 ^f	137
Diamide (0.15 mmol/l)	30	normal	2.5 $\pm$ 0.2	6.5 $\pm$ 0.6 ^g	160
		denervated	2.1 $\pm$ 0.2	3.2 $\pm$ 0.4 ^d	52
H ₂ O ₂ (1 mmol/l)	10	normal	2.4 $\pm$ 0.1	5.8 $\pm$ 0.2 ^g	142
		denervated	2.4 $\pm$ 0.2	5.4 $\pm$ 0.3 ^g	125
Vitamin K ₅ (100 $\mu\text{mol/l}$)	30	normal	2.0 $\pm$ 0.1	5.5 $\pm$ 0.5 ^g	175
		denervated	2.3 $\pm$ 0.3	6.4 $\pm$ 0.3 ^g	178
Anoxia	b	normal	2.0 $\pm$ 0.3	7.0 $\pm$ 0.4 ^f	250
		denervated	1.6 $\pm$ 0.2	5.4 $\pm$ 0.3 ^g	238
2:4-Dinitrophenol (0.5 mmol/l)	10	normal	2.4 $\pm$ 0.2	7.1 $\pm$ 0.6 ^f	196
		denervated	1.8 $\pm$ 0.2	5.6 $\pm$ 0.5 ^f	211
Cooling	c	normal	2.4 $\pm$ 0.3	6.1 $\pm$ 0.2 ^g	154
		denervated	2.1 $\pm$ 0.1	5.7 $\pm$ 0.2 ^g	171
Mannitol (200 mmol/l)	30	normal	2.2 $\pm$ 0.4	7.5 $\pm$ 0.7 ^e	241
		denervated	1.6 $\pm$ 0.2	5.7 $\pm$ 0.1 ^g	256
EDTA (5 mmol/l)	5	normal	2.4 $\pm$ 0.2	5.3 $\pm$ 0.4 ^g	121
		denervated	2.1 $\pm$ 0.1	5.8 $\pm$ 0.2 ^f	176

Results are expressed as mean $\pm$ SEM of five to seven determinations.

With the exceptions noted below, soleus muscle pairs were preincubated for 30 min at 37 °C under aerobic conditions. At this point the agents shown were added to one muscle and the incubation continued for the times shown in the second column.

^a 30 mmol/l xylose was added to one muscle together with the radioactive test sugars. To avoid complication due to the effect of hyperosmolarity, an equivalent amount of NaCl was omitted from the medium; ^b Muscles were preincubated for 60 min at 37 °C under either 95% O₂-5% CO₂ or 95% N₂-5% CO₂; ^c Muscle pairs were preincubated for 30 min at 37 °C; one muscle from each pair was then incubated for 3 h at 0 °C. Xylose uptake was measured at 37 °C.

Activated versus basal: ^d $p < 0.05$; ^e $p < 0.01$; ^f $p < 0.005$; ^g $p < 0.001$

denervated muscle until the insulin concentration exceeded 1 U/l. Insulin, de-activated by exposure to urea and dithiothreitol (see Methods), was without effect on xylose transport in normal or denervated muscles. Control experiments, where muscles were exposed to the same concentration of urea and dithiothreitol as present in the deactivated insulin, established that these agents did not affect either basal or insulin-stimulated sugar transport.

The experiment shown in Figure 3 examined ¹²⁵I-insulin binding by normal and denervated soleus muscle. Muscles were incubated for 90 min at 25 °C in the presence of ¹²⁵I-insulin (0.17 nmol/l) and various concentrations of unlabelled insulin (up to 3.3 $\mu\text{mol/l}$). The competitive binding curves thus obtained are shown in Figure 3 A and the Scatchard plots derived therefrom are shown in Figure 3 B. Denervation did not affect ¹²⁵I-insulin binding. Specific ¹²⁵I-insulin binding, calculated from the binding values at 0.17 nmol/l, was 73 $\pm$ 5 fmol ¹²⁵I-insulin bound/g normal muscle and 65 $\pm$ 4 fmol ¹²⁵I-insulin bound/g denervated muscle ($n = 10$, NS).

Denervation has variously been reported both to stimulate and to depress the uptake of the amino acid analogue, α -aminoisobutyrate, by muscle [15, 16]. We found that denervation did not affect basal α -aminoisobutyrate uptake in soleus muscle (Table 1). However,

the stimulatory effect of insulin on α -aminoisobutyrate uptake was reduced from 117% in normal muscle to 25% in denervated muscle. We also examined glucose-independent activation of glycogen synthase by insulin in normal and denervated muscle. The total activity of glycogen synthase, measured in the presence of 20 mmol glucose-6-phosphate/l was reduced from 0.63 $\pm$ 0.03 U/g muscle ($n = 6$) in normal muscle to 0.40 $\pm$ 0.05 U/g ($n = 6$) in denervated muscle. Nevertheless, the percentage of glycogen synthase in the active form was the same in both muscle groups following incubation under basal conditions, or in the presence of insulin (Table 1).

To define more precisely the nature of the defect involved, we examined the effect of denervation on the action of a number of other agents which have been shown to stimulate sugar transport. Apart from increasing the concentration of xylose itself, the other agents used for these experiments were concanavalin A [17–19], the oxidants, diamide, H₂O₂ and vitamin K₅ [20, 21], anoxia and 2:4-dinitrophenol [10], cooling [22], hyperosmolarity (200 mmol mannitol/l) [23] and EDTA [24]. The natural variation between groups of rats tends to complicate any comparison between nor-

mal and denervated muscles in terms of absolute uptake rates. A more suitable basis for comparison is the percentage stimulation achieved in each group. Denervation did not affect the ability of the sugar transport system to respond when the concentration of xylose was increased from 10 to 40 mmol/l (Table 2). Similarly, denervation did not suppress the stimulatory effects of concanavalin A, H_2O_2 , vitamin K_5 , anoxia, 2:4-dinitrophenol, cooling, hyperosmolarity or EDTA. Denervation reduced the stimulatory effect of diamide from 160% in normal muscle to 52% in denervated muscle.

Discussion

From the observed effects of denervation on insulin-stimulated sugar transport in diaphragm, Buse and Buse [9] concluded that insulin-sensitivity was governed by the activity of the muscle. Support for this theory comes from more recent studies showing that glucose tolerance is impaired in bedridden patients [25] and in patients with spinal cord injuries [26] and is improved by a programme of exercise training [27].

The studies reported in this paper confirm and extend the earlier observations of Buse and Buse [8, 9]. Whereas they found that denervation abolished insulin action in the diaphragm, our experiments have shown that denervation does not abolish the action of insulin on sugar transport, but rather induces a massive resistance to the hormone. The apparent contradiction between the effect of denervation on diaphragm and soleus muscle is easily explained in terms of the insulin concentration used by Buse and Buse (1 U/l). We also found that there was no effect of 1 U insulin/l on sugar transport in denervated soleus muscle; it was only when higher concentrations of insulin were used that denervated muscle showed any response to the hormone.

Perhaps one of the most important facts to emerge from the studies of Buse and Buse [8] was that denervation did not affect the ability of the muscle to bind ^{125}I -insulin, thus localizing the defect to the post-receptor action of the hormone. However, because their work predated the current practice of measuring specific (i.e. displaceable) as distinct from non-specific (non-displaceable) insulin binding, their values would have included both specifically- and non-specifically-bound insulin. Using a method which differentiates between specific and non-specific binding, we have confirmed that denervation does not affect specific binding of ^{125}I -insulin by soleus muscle.

Denervation inhibited the stimulatory effect of insulin on amino acid transport to the same extent as its inhibition of insulin-stimulated sugar transport (Table 1; [15]). Conversely, the ability of insulin to activate glycogen synthase (by the glucose-independent mechanism) was unaffected by denervation. This, together with the ^{125}I -insulin binding data, indicate that the mechanism whereby insulin binds to its receptor and generates

an intracellular mediator [5] is not impaired in denervated muscle. Thus, the mechanism concerned with the activation of the intracellular enzyme, glycogen synthase, differs from that involved in the activation of the two membrane transport systems. Dissociation between insulin's action on membrane systems and intracellular enzyme systems can also be demonstrated using puromycin [28], N-ethylmaleimide [29] and neuraminidase [30]. The action of insulin on intracellular enzyme systems is believed to be effected through the release of a low-molecular-weight mediator protein from the plasma membrane [5-7]. The fact that insulin's intracellular actions can be dissociated from its effect on membrane transport systems could suggest, *prima facie*, that those actions of insulin which involve membrane-localized systems are mediated through some mechanism other than the 'intracellular' messenger.

Because denervation inhibits insulin-stimulated sugar transport at the post-receptor level, it can serve as a probe to examine the coupling of the insulin receptor to the sugar transport system. Nine of the ten insulinomimetic agents shown in Table 2 were still able to activate sugar transport in denervated muscle to the same extent as in normal muscle. This suggests that the defect in denervated muscle is at some early post-receptor step. Two of the results in Table 2 deserve further comment: first, the insensitivity to denervation of the stimulatory effect of concanavalin A. Concanavalin A is one of a number of plant lectins which has been shown to stimulate glucose transport in adipocytes [17, 18]; to our knowledge the effect of concanavalin A on muscle sugar transport has not hitherto been reported. Cuatrecasas [31] has suggested that the insulin-like actions of concanavalin A in the adipocyte could result from the binding of the lectin to the insulin receptor, thereby triggering an 'insulin' signal. If this were so, it could be expected that the effect of the lectin would be depressed by denervation. The ability of concanavalin A to stimulate sugar transport in denervated muscle suggests that its action could be triggered through some other mechanism.

The second point which arises from the results shown in Table 2 concerns the actions of diamide, hydrogen peroxide and vitamin K_5 . These are members of a group of oxidants which have been shown to stimulate sugar transport in adipocytes [21]. Sorensen et al. [32] have reported that hydrogen peroxide stimulates sugar transport in rat soleus muscle. Apart from this and the results reported in this paper, the effect of oxidants on muscle sugar transport has not otherwise been studied. According to Czech [20], these agents all activate sugar transport in the adipocyte because of their ability to oxidize some essential sulphhydryl group(s). It is surprising that denervation inhibits the stimulatory effect of diamide on muscle sugar transport, but does not affect the action of hydrogen peroxide and vitamin K_5 . If, as has been assumed, the stimulatory effect of all these oxidants involved the same metabolic step,

then it seems reasonable to expect that all should have been affected similarly by denervation. The anomalous behaviour of diamide in this respect suggests that its effect on sugar transport is mediated separately from and prior to the effects of hydrogen peroxide and vitamin K₅. Beyond the simple observation that diamide stimulates sugar transport in the adipocyte, the underlying mechanism of action of this agent has not been further explored. These studies have shown that the action of diamide differs from that of the other oxidants; the precise nature of the action of diamide and the other oxidants remains to be determined.

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