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# Effect of chronic treatment with Vitamin E on endothelial dysfunction in a Type I in-vivo diabetes mellitus model and in vitro.

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**Running title: Vitamin E in diabetes and hyperglycemia**

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Abbreviations:

| | |
|---|---|
| NO | nitric oxide |
| ATP | adenosine triphosphate |
| LNNA | L-$N^G$-Nitro-arginine |
| GTN | glyceroyltrinitrate |
| Dil-Ac-LDL | 1,1'dioctadecyl3,3,3'33'-tetramethylindo-carbocyanine-acetylated low density lipoprotein |
| PBS | phosphate-buffered saline |
| H.E. | hematoxylin eosine staining |
| oxy-Hb | oxy-hemoglobin |
| Met-Hb | met-hemoglobin |
| FCS | fetal calf serum |
| PARP | poly(ADP-ribose)-polymerase |

**Abstract**

Diabetes mellitus often leads to generalized vasculopathy. Because of the pathophysiological role of free radicals we investigated the effects of vitamin E. 28 rats were rendered diabetic by streptozotocin injection and were fed either with a diet with low (10 mg/kg chow), medium (75 mg/kg chow) or high vitamin E (1300 mg/kg chow). 9 age-matched non-diabetic rats receiving 75 mg vitamin E/kg/chow served as controls. After 7 months mesenteric microcirculation was investigated. Smooth muscle contractile function was not altered in diabetic vs. non-diabetic vessels. Endothelial function was significantly reduced in diabetics: relaxation upon 1 µM acetylcholine was reduced by 50% in diabetics with medium and high vitamin E diet. In vitamin E-deprived rats a complete loss of endothelium-dependent relaxation was observed and instead acetylcholine elicited vasoconstriction. LNNA-induced vasoconstriction was reduced in small arteries in diabetics which was not prevented by vitamin E, but was aggravated by vitamin E deprivation. In a subchronic endothelial cell culture model cells were cultivated with 5 or 20 mM D-glucose for an entire cell culture passage (4 days) with or without vitamin E (20 mg/l vs. 0.01 mg/l). Hyperglycemia led to significant reduction in basal and ATP-stimulated NO-production. Hyperglycemia-induced reduction in basal NO-release was significantly prevented by vitamin E, while reduction in stimulated NO-release was not influenced. NADPH diaphorase activity was reduced by 40% by hyperglycemia which was completely prevented by vitamin E. We conclude, that (a) vitamin E has a potential to prevent partially from hyperglycemia-induced endothelial dysfunction, (b) under in-vivo conditions vitamin E deficiency enhanced diabetic endothelial dysfunction dramatically, (c) positive effects of vitamin E may be attenuated with longer disease duration.

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An intriguing problem in internal medicine is the development of generalized angiopathy in the course of diabetes mellitus. This is associated with the occurrence of endothelial dysfunction (Oyama et al., 1986; Cameron & Cotter, 1992; Pieper & Peltier, 1995; Pieper et al., 1997; Olbrich et al., 1996). It is known that hyperglycemia can lead to changes in endothelial nitric oxide production or release: short-term or subacute exposition to high D-glucose was shown to result in enhanced NO release (Graier et al., 1993, 1996) while chronic exposure during an entire cell culture passage leads to reduced NO-release (Olbrich et al., 1999) and diminished calcium signals (Salmeh & Dhein, 1998). It was hypothesized by these authors and by others (Pieper et al., 1997; Du et al., 2000; Nishikawa et al., 2000) that free radicals may be involved in the pathophysiology of endothelial dysfunction. Meanwhile there is broad evidence supporting an important pathophysiological role for free radicals in diabetic vasculopathy (Spitaler & Graier, 2002). Thus, a central role for superoxide overproduction in the pathobiochemistry of the main pathways of hyperglycemia-related changes, i.e. activation of protein kinase C, accumulation of advanced glycation end products, increased flux of glucose through the aldose reductase pathway, has been shown (Nishikawa et al., 2000). Recently, Rösen and colleagues (1998) showed that vitamin E can prevent from reduction in endothelial NO release in diabetic rat heart. They postulated that during hyperglycemia the endothelium may be deprived with l-arginine or that an increased NO level may be inactivated by increased levels of free radicals (Rösen et al., 1998). In addition, Cinar and colleagues (2001) showed in a 3 months model of diabetes a protective effect of 1000 mg vitamin E/ kg chow against endothelial dysfunction. Similarly, a protective effect was shown in a mouse model (Göcmen et al., 2000), or in 2 months rat model (Keegan et al., 1995). In support of these studies Kunisaki and colleagues (1995) showed in a rat model that diabetes led to enhanced PKC translocation and increased diacylglycerol formation which both could be prevented by 2 weeks vitamin E treatment in retinal vascular endothelial cells. In another diabetes rat model, vitamin E attenuated but did not completely prevent diabetes-induced endothelial dysfunction (Karasu et al., 1997a). 2 months dietary vitamin E supplementation in the diabetic rat reduced lipid peroxide levels (Karasu et al., 1997b). However, it remained unclear whether such positive effects of vitamin E might be attenuated in models with longer duration of the disease.

While vitamin E treatment was shown to prevent from thromboxane overproduction in diabetic patients (Gisinger et al., 1988), which has been suggested to indicate a possible

vasoprotective effect, and while improvement of endothelial function in diabetic patients by vitamin E has been found (Skyrme-Jones et al., 2000), others failed to demonstrate a positive preventive effect of additional vitamin E in diabetes mellitus (Nickander et al., 1994; Dagenais et al., 2001; Lonn, 2001; Lonn et al., 2001).

Since diabetes is a chronic disease and since most animal studies investigated a considerably short duration of diabetes of 1 to 3 months, we wanted to know whether vitamin E influences vascular function in a long-term diabetes rat model of 7 months duration which is approximately a quarter of the normal life span of the rat. Furthermore, we wanted to elucidate the effects of vitamin E in a subchronic cell culture model of hyperglycemia previously established by our group (Salameh & Dhein, 1998) with a duration of an entire cell culture passage (and not only 24 hours as often used). Thus, the aims of our study were to test whether in an in-vivo type I diabetes mellitus rat model vitamin E deprivation might worsen the development of endothelial dysfunction and whether vitamin E supplementation (medium and high) might prevent. Furthermore, it should be investigated whether in a subchronic cell culture model chronic hyperglycemia leads to reduced endothelial NO production and whether this can be prevented by vitamin E.

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## Methods

### In-vivo Study

All experiments were performed according to the ethical rules of the Council for International Organization of Medical Science and the German laws for animal welfare.

We used a streptozotocin rat model of type I diabetes with a duration of diabetes of 7 months as described (Dhein et al., 2000). Four experimental groups were investigated (a) control animals without treatment (normal vitamin E alimentation: 75 mg/kg chow) (n=9), (b) diabetic animals without treatment (normal vitamin E alimentation; 75 mg/kg chow) (n=6), (c) diabetic animals receiving vitamin E enriched diet (1300 mg/kg chow) (n=8), (d) diabetic animals receiving vitamin E deprived diet (0.55 mg/kg chow) (n=6). Vitamin E was supplied as α-tocopherol. Vitamin E plasma levels were 10±1 mg/l in animals receiving medium vitamin E alimentation, 19±2 mg/l in animals receiving vitamin E enriched diet and 2.2±1 mg/l in rats receiving vitamin E deprived diet. For comparison normal rat diets are supplemented with vitamin E ranging from 30 to 200 mg / kg chow (so that 75 mg /kg chow resembles a normal rat diet) (Lehr et al., 1999).

For induction of diabetes mellitus, six-week-old male Wistar Kyoto rats (140±20g) were rendered diabetic by intraperitoneal (i.p.) injection of streptozotocin (STZ, 60 mg/kg bodyweight) as described (Dhein et al., 2000). Two weeks after the induction of diabetes mellitus, animals were randomized to the treatments, i.e. no treatment, vitamin E enriched diet or vitamin E deprived diet. The animals did not receive an antidiabetic treatment.

*Vascular function*

For functional measurements of smooth muscle and endothelial function a mesenteric loop was isolated with the appertaining intestine (length: 8 cm) according to the technique described earlier (Dhein et al., 1992, 2000; Olbrich et al., 1996). The mesenteric artery was canulated and perfused with oxygenated Tyrode's solution ($Na^+$ 161.02, $K^+$ 5.36, $Ca^{++}$ 1.8, $Mg^{++}$ 1.05, $Cl^-$ 146.86, $HCO_3^-$ 23.80, $H_2PO_4^-$ 0.42, glucose 10.00 mM, pH adjusted to 7.4; gassed with 95% $O_2$ and 5% $CO_2$). A 8 cm loop of the small intestine was ligated, and all side branches of the mesenteric vessels were sealed by ligation so that an isolated mesenteric fold with the appertaining intestine and the perfusing arterial network was prepared. This preparation was fixed to a perfusion system with a constant perfusion pressure of 70 cm $H_2O$, which corresponds to the actual physiological perfusion pressure in the mesenteric artery in this model. 10 canulas were inserted into the intestine to provide drainage. With the help of a

microscope (C.Zeiss, Germany) and a video camera (Sony, Japan), which was mounted behind the ocular of the microscope, the mesenteric vessels were displayed on a monitor (Sony, Japan) The total magnification was 240-fold. In the course of the experiments pictures of the arteries were recorded. Vessel diameters were determined during the experiment directly on the screen and after the experiments re-evaluated in the digitalized pictures using a frame grabber board (Data Translation, Marlboro, USA) with JAVA software (Jandel Scientific, Erkrath, Germany). Vessel diameter was assessed by analyzing the first derivative of the gray level along a cross sectional line (orthogonal to the vessels longitudinal axis). The distance between the extremata corresponds to the vascular diameter. We classified microvessels according to the generation theory of Ley and colleagues (1986) as G1-vessels, which are the branch perfusing the isolated loop. The subsequent branches were classified as G2, G3 and G4 vessels the latter being those vessels at the border between mesenterium and gut. More details of the method are given by Olbrich et al. (1999).

After an equilibration period of 60 minutes to achieve a constant resting tone, vessels were preconstricted by infusion of 70 mM KCl (20 min) followed by treatment with KCl (70 mM) and 1 µM glyceroyltrinitrate (GTN) (20 min). After washout and reaching the preconstriction tone with 70 mM KCl alone vessels were perfused with 70 mM KCl and 1 µM acetylcholine (20 min). After washout the vessels were exposed to 3 µM L-$N^G$-nitro-arginine (20 min) for inhibition of NO synthase. KCl was used as constrictor, since this was also used by others in diabetic rat mesenterial vessels (Ralevic et al.,m 1995; Misurkski et al., 2001; Van Buren et al., 1998) and was reported by these authors to be only weakly affected by diabetes.

We wanted to use a constrictor which is not or only weakly affected by diabetes. In accordance with the above mentioned literature KCl seems to be suitable. The response to methoxamin or other constrictors acting via receptors might be altered in diabetes if the signal transduction pathways or the receptors themselves may be affected (e.g. by advanced glycation end products). Thus, Van Buren et al. (1998) describe that the sensitivity for norepinephrine is altered in diabetes while the sensitivity for KCl is not (or only weakly). Similarly, methoxamine-induced contraction is attenuated (Misurski et al., 2001). However, it should be mentioned that EDHF-dependent relaxations might be affected by KCl.

**Cell Culture Study**

*Cell isolation and culture*

In previous investigations we established a subchronic cell culture model of hyperglycemia-induced endothelial dysfunction (Salameh & Dhein, 1998) using porcine aortic endothelial cells exposed to hyperglycemia for an entire culture cell passage (4 days). Therefore, porcine aortic endothelial cells were isolated and cultured according to Rosenthal and Gotlieb (1990) as previously described (Salameh et al., 1997). Briefly, the endothelial cells were harvested from porcine thoracic aorta using 1 mg/ml dispase, seeded (100,000 cell/$cm^2$) in plastic-9.6 $cm^2$ Petri dishes (Nunc, Wiesbaden, Germany) and cultured with M199 at 37°C, saturated humidity and 5% $CO_2$. After reaching confluence the cells were passaged and seeded again. Purity of the cell culture was tested by uptake of Dil-Ac-LDL (1,1'dioctadecyl3,3,3'33'-tetramethylindo-carbocyanine-acetylated low density lipoprotein) (Voyta et al., 1984) and, for detecting contaminating smooth muscle cells, by staining of α-smooth muscle actin.. At the start of the third passage, the cells were submitted to the various treatments. The different experimental protocols were carried out with cells of the same cell line for intraindividual control (i.e. all cells were derived from the same aorta) at the moment when they were seeded for third passage.

Thus we used the following experimental groups:

5 mmol/l D-glucose alone (n=9)

5 mmol/l D-glucose plus 15 mmol/l L-glucose (for osmotic control) (n=6)

5 mmol/l D-glucose plus 20 mg/l vitamin E (α-tocopherol) (n=6)

20 mmol/l D-glucose (high D-glucose, „hyperglycemia") (n=6)

20 mmol/l D-glucose plus 20 mg/l vitamin E (α-tocopherol) (n=6)

The specific treatment of the different control or experimental groups started at the third passage and lasted until the cells had reached confluence (3-4 days). As before, the medium was changed 3 times a week.

*Histological studies*

For hematoxylin-eosin staining (H.E.), endothelial cell monolayers were washed 3 times with phosphate-buffered saline [(PBS) containing (m*M*): NaCl, 137; KCl 2.68; $Na_2HPO_4$, 8.1; $KH_2PO_4$, 1.47; buffered at pH 7.4] and fixed in paraformaldehyde solution (4% paraformaldehyde in PBS) 30 min at room temperature. After we removed the fixative, the cells were stained in 1 % hematoxylin and 0.5% eosin following classical protocols and embedded in Karion F (Merck, Darmstadt, Germany).

*NADPH-Diaphorase staining.* NADPH-diaphorase reaction was carried out according to Hope et al. (1991). In brief, confluent monolayers were fixed in 4% paraformaldehyde for 30 min at room temperature and incubated in the staining solution, containing 0.5 m*M* nitro-blue tetrazolium, 1 m*M* ß-NADPH, 0.2% Triton X-100, 50 m*M* Tris and 75 m*M* NaCl, buffered at pH 8.0, for 20 h at 37°C. Thereafter, the preparations were washed three times in PBS and embedded in Karion F.

For quantitative analysis, the histologic specimens were viewed through a microscope (Leitz, Wetzlar, Germany) and a Sony videocamera (Video 8, CCD-V90E sensitivity 7 lux, Sony, Tokyo, Japan), which communicated with a video framegrabber board (QuickCapture Board DT 2855; Data Translation Inc., Marlboro, MA, USA) and a common PC system. The pictures were converted digitally and could be analyzed by means of the picture-analyzing system JAVA (Jandel video analysis software; Jandel Scientific, Erkrath, Germany). This software allowed the determination of NADPH-diaphorase activity by evaluating the intensity of the blue color staining for NADPH-diaphorase (reduced nitro-blue tetrazolium) of approximately 100 cells within an area of interest in each cell line. The number of giant cells (cells 3-4 times larger than normal endothelial cells) was counted under the microscope at 400x magnification within a visual field of 40 $mm^2$, arbitrary marked.[x visual fields per experimental series (i.e. y visual fields per cell line)].

*Measurement of nitric oxide release*

To characterize endothelial function, we measured the NO release spectrophotometrically (UV-DU-7500 Beckmann, Munich, Germany) under basal conditions and after stimulation with adenosine triphosphate (ATP 1 m*M*) using the methemoglobin assay (Feelisch & Noack, 1987), based on the rapid oxidation of reduced methemoglobin (oxy-Hb, oxyhemoglobin, $Fe^{2+}$) to methemoglobin (Met-Hb, $Fe^{3+}$) by nitric oxide. The suitability and specificity of this assay has been demonstrated previously (Kelm et al., 1997). We monitored increasing amounts of methemoglobin versus oxyhemoglobin by means of the difference spectrum (Feelisch & Noack, 1987). The bioassay was calibrated as described previously (Feelisch & Noack, 1987; Kelm et al., 1988). We found an extinction coefficient e of 39 $mM^{-1}$ $cm^{-1}$ which nearly identical to that described by Feelisch and Noack (1987). After reaching confluence the PAEC were washed three times with HEPES-buffer, [composed of (m*M*): NaCl 145.0; KCl 5.0; $CaCl_2$ 2.5; $MgCl_2$ 1.0, HEPES 10.0; D-Glucose 5.0] at pH 7.4, 37°C., preincubated with 4 ml HEPES-buffer for 20 min, 37°C, and supplemented with oxy-Hb-

solution (4 µmol/l). After an equilibration period of 50 min, 1 mM ATP was added and subsequently NO release was recorded for 40 minutes, cycling time was 10 minutes, 37°C, for each cell culture condition intraindividually. To obtain the actual formation of methemoglobin, representing the NO release by PAEC, we subtracted the spontaneously occurring formation of methemoglobin, determined from a cell free incubation solution from the measurements.

**Chemicals**

All chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) except for Dil-Ac-LDL (1,1'-dioctadecyl3,3,3',33'-tetramethylindo-carbocyanine-acetylated low density lopoprotein), which was obtained from Paesel+Lorei (Frankfurt, Germany), dispase was obtained from Boehringer Mannheim, Germany, nitro-blue tetrazolium was purchased from Biomol (Hamburg, Germany), KCl were obtained from Merck (Darmstadt, Germany), ACh and heparin from Serva (Heidelberg, Germany). All cell culture media and fetal calf serum (FCS) were obtained from Sigma (St. Louis, USA), the cell culture material was obtained from Nunc (Wiesbaden, Germany). All chemicals were of analytic grade and were dissolved in bidistilled water if not stated otherwise.

**Statistical analysis**

For statistical analysis, a two-factorial analysis of variance (ANOVA) was performed. If Anova indicated significant differences or significant interactions between disease and treatment, the data were further analyzed with a post-hoc Tukey-HSD test. For the statistical analysis, we used Systat for windows software, version 5.02 (Systat, Evanston, IL). Differences were considered significant if $p<0.05$.

## RESULTS

### In Vivo Study

Streptozotocin injection caused diabetes mellitus within two weeks and blood glucose levels of >18 mM in all diabetic groups (no differences between the three diabetic groups). In non-diabetic age-matched control animals we found blood glucose levels ranging from 3.5 to 5.6 mM. Body weight was reduced in diabetic animals (228±10 (d.m.), 237±3 (d.m.+Vitamin E), 199±7 g (d.m. - Vitamin E)) as compared to non-diabetic age-matched controls (418±8 g). Plasma vitamin E levels were 10±1 mg/l (animals receiving 75 mg/kg chow), 19±2 mg/l (animals receiving vitamin E enriched diet) and 2.2±1 mg/l (rats on vitamin E deprived diet) (see table 1).

With regard to the vascular parameters, the initial diameters of the mesenterial microvessels under resting conditions were somewhat enlarged in diabetic animals (see table 2). This was not influenced by the treatment.

Regarding smooth muscle vascular function we found a reduction in vessel diameter by 70 mM KCl by 44 to 29 % in non-diabetics. In G1, G2 and G3 vessels this was not significantly altered in diabetics. In G4 vessels KCl-induced constriction was slightly attenuated in diabetics. Additional vitamin E treatment or vitamin E deficiency did not influence KCl contraction (see figure 1A).

GTN application in all vessels lead to significant vasodilation which was diminished in diabetics. Additional treatment with vitamin E did not influence GTN-induced vasorelaxation. However, in vitamin E deprived rats we found significantly decreased GTN-induced relaxation as compared to untreated diabetics (see figure 1B).

Acetylcholine induced vasorelaxation in all vessels in non-diabetic rats. In diabetic rats this ACh-induced relaxation was significantly decreased, which was not influenced by vitamin E treatment. However, in vitamin E deficiency we found complete abolition of ACh-induced relaxation. In contrast, ACh in these rats induced slight vasoconstriction (figure 1C).

Finally, we applied L-$N^G$-nitro-arginine (LNNA), which resulted in a significant vasoconstriction of all vessels. The LNNA-induced vasoconstriction reached 8-14% of the KCl-constriction. In diabetic rats this LNNA-induced constriction was significantly reduced in G3 and G4 vessels. This was not influenced by vitamin E treatment. However, in vitamin E deficiency we found significant attenuation of LNNA-induced vasoconstriction in all vessels (figure 1D).

**Cell Culture Study**

**NO-release**

Cells reached confluence after 3.5±0.5 days without differences between the groups. We found the typical difference spectrogram for Met-Hb vs.oxy-Hb with an isobest at 412±1 nm and maximum extinction at 402±1 nm as described (Feelisch & Noack, 1987). Under basal condition, using normal cells there was a slow increase in extinction as can be seen from 2A during the first 50 min indicating increasing formation of Met-Hb and release of NO. Stimulation with 1 mM ATP led to a further increase in extinction. In cells, which were grown under hyperglycemic conditions, however, basal formation of Met-Hb was significantly reduced (fig. 2B, fig. 3). ATP-stimulated formation of Met-Hb was also clearly diminished (figure 3). Quantitatively we found basal release of 80±20 pMol * 1 Mio cells$^{-1}$*10 min$^{-1}$, which was significantly diminished to 40±10 pMol * 1 Mio cells$^{-1}$*10 min$^{-1}$ ($p<0.05$) (figure 3). ATP-stimulated NO-release was significantly reduced from 130±20 (normal cells) to 92±10 pMol * 1 Mio cells$^{-1}$*10 min$^{-1}$ in hyperglycemic cells ($p<0.05$).

The reduction in basal NO-release in hyperglycemic cells was significantly antagonized by vitamin E (fig. 3). However, stimulated NO-release was not affected by vitamin E treatment: in vitamin E treated cells stimulation with 1 mM ATP resulted in 155±20 pMol * 1 Mio cells$^{-1}$ *10 min$^{-1}$ in normoglycemic and 98±40 pMol * 1 Mio cells$^{-1}$ * 10 min$^{-1}$ in hyperglycemic cells. Thus, there was no antagonization of the glucose effect by vitamin E regarding stimulated NO-release (figure 3).

The osmotic control using additional 15 mM L-glucose revealed that there was no alteration by 15 mM L-glucose as compared to normoglycemia (figure 4).

**Histology**

For further characterization of the glucose effects we investigated the cultured cells histologically. In all cell lines investigated, >99% of the tested cells incorporated Dil-Ac-LDL with no significant differences between the two experimental groups. The content of contaminating smooth muscle cells was <0.1%. The high D-glucose-treated cell lines did not show any promotion of smooth-muscle cell growth in culture. The three control groups (5 m*M* D-glucose without any treatment or with 20 mg/l vitamin E or with 15 m*M* L-glucose) exhibited no significant differences concerning their cell morphology. Moreover, the number

of giant cells and the NADPH-diaphorase activity per cell were not different between the control groups.

Morphological analysis of the cells revealed that hyperglycemia led to an enhanced number of giant cells which was significantly increased from 7±2 to 17±3 under treatment with high D-glucose ($p<0.05$). In vitamin E treated cells this increase in giant cell number was significantly prevented ($p<0.05$). Thus, in vitamin E treated normoglycaemic cells we found 6±3 and in hyperglycemic vitamin E treated cells 7±3.

Furthermore, the high D-glucose influence on PAEC was characterized by a reduction in histochemical NADPH-diaphorase activity. Considering the intensity of reduced NBT in relationship to the total area of interest, we found an intensity of 53.6±2.6 densitometric units in normoglycemic cells versus 30.4±2.5 densitometric units in hyperglycemic cells ($p<0.05$) (figure 5). Treatment with vitamin E significantly prevented from this reduction in NADPH-diaphorase activity: 47.3±3.5 versus 47.6±2.5 densitometric units (n=8).

## Discussion

*In vivo Study*

Taken together our data show that smooth muscle contractile function was not altered by diabetes mellitus as evident from nearly unchanged KCl-induced contractions. However, relaxation to GTN and ACh were significantly attenuated. This diabetes-induced deficit in relaxation was not influenced by high vitamin E treatment, but was further enhanced by vitamin E deficiency. It is well known that GTN-induced vasorelaxtion depends on glutathion-dependent release of NO from S-nitrosothiol-derivatives of GTN. Since in diabetes deprivation of glutathion occurs in vascular tissue (Kinalski et al., 2000) it is reasonable, that in our study relaxation to GTN was attenuated in diabetic rats. In contrast, ACh-induced relaxation depends on release of NO from functional vascular endothelium. As in previous studies (Olbrich et al., 1996, 1999; Dhein et al., 2000) ACh-induced vasorelaxation was diminished in diabetics indicating endothelial dysfunction. Most interestingly, while this was not affected by high vitamin E treatment, vitamin E deficiency further diminished vasorelaxant response to GTN and completely abolished relaxation to ACh with a reversal of the ACh-response so that vasoconstriction occurred. It should be mentioned, that vitamin E deprivation for 4 to 12 months itself impairs endothelial relaxation while smooth muscle responses are not affected (Rubino & Burnstock, 1994; Rubino et al., 1995; Ralevic et al., 1995). However, these investigators observed only reduced vasorelaxation in vitamin E deprivation. Thus, both diabetes mellitus and vitamin E deprivation can lead to an impairment of endothelial function. The combination of diabetes and vitamin E deprivation, however, seems to be even more deleterious leading to complete loss of endothelial function so that ACh elicits vasoconstriction. This is –to our best knowledge- the first report showing this dramatic change in the response to acetylcholine.

In accordance with the impaired ACh-induced relaxation we found vasoconstriction in response to the NO-synthase inhibitor LNNA, which were significantly attenuated in vitamin E deficiency. The vasoconstriction to LNNA may be interpreted as the consequence of inhibition of basal NO release which was more prominent in G3 and G4 vessels. The results indicate that there was less basal NO production in diabetic animals in G3 and G4 vessels and further attenuation of basal NO release in vitamin E deficiency. As an explanation, it has been argued that in vitamin E deficiency with concomitant hyperglycemia there is enhanced production of free radicals which can interact with NO leading to inactivation of NO (Pieper et al., 1997; Stockklauser-Farber et al., 2000). Moreover, our finding of a diminished GTN-

induced relaxation in vitamin E deficient diabetic rats can be explained by the observation that reduction in glutathion levels occurs in vitamin E deficiency and is further enhanced in concomitant diabetes mellitus (Nickander et al., 1994).

These results may indicate that with 75 mg vitamin E/kg chow there was no deficit in vitamin E and that high vitamin E treatment does not improve vascular function in this long-term model. However, it should be mentioned that high vitamin E intake may further impair endothelial function as was shown in a 1 month rat diabetes model (Palmer et al., 1998). Our results would be in accordance with the findings of the HOPE study (Dagenais et al., 2001). However, our data clearly indicate that vitamin E deficiency dramatically worsens the situation and aggravates diabetes-induced vascular dysfunction in accordance with another clinical study (Salonen et al., 1995) or animal studies (Vanucchi et al., 1999, Nickander et al., 1994). Since vitamin E is a well known radical scavenger, this may further support the hypothesis of a pathophysiological role for free radicals in diabetic vascular dysfunction.

*In vitro Study*

In order to investigate the results of the in-vivo study in more detail, we exposed endothelial cells subchronically to hyperglycemia for an entire culture passage, which resulted in reduced NO release. This could partially be prevented by vitamin E: only the hyperglycemia-induced reduction in basal NO release was prevented by vitamin E while reduction in stimulated NO release was not prevented.

Hyperglycemia-induced reduction in basal and stimulated NO-release could be explained by reduced arginine supply or reduced access to the intracellular arginine pool (Pieper & Peltier, 1995; Closs et al., 2000; Hardy & May, 2002) or by reduced eNOS activity following mitochondrial superoxide overproduction which can lead to O-linked N-acetylglucosamine modification of eNOS (Du et al., 2001) via activation of hexosamine pathway (Du et al., 2000). Igarashi and Michel (2001) suggested that the production of reactive oxygen species leads to activation of the glucosamine pathway by activation of glutamin-fructose-6-phosphate amidotransferase GFAT finally leading to O-glycosylation of eNOS. The latter has been shown in a cell culture model similar to ours after chronic exposure (2 days) to high D-glucose levels (Du et al., 2001). eNOS expression, however, is not altered in hyperglycemia (Stockklauser-Farber et al., 2000). However, Rösen and coworkers (1996) showed increased eNOS activity which was assumed to compensate for enhanced NO-inactivation by free radicals in the heart, although in the recent study these investigators did not find enhanced

eNOS activity (Stockklauser-Farber et al., 2000). Another explanation could be a reduction in NADPH supply (a necessary cofactor for eNOS). However, others showed that NADPH is not altered in hyperglycemia (Asahina et al., 1995) but is reduced if additional oxidative stress is present. On the other hand, Soriano and colleagues (2001a) demonstrated in a streptozotocin mouse model, that vascular NAD(+), NADPH and ATP levels were decreased. These authors showed that activation of poly(ADP-ribose)-polymerase (PARP) by oxidant-induced DNA strand breakage (via glucose-induced free radical generation (Soriano et al., 2001b)) is involved in diabetic endothelial dysfunction. Most interestingly, endothelial (Soriano et al., 2001a) and cardiac (Pacher et al., 2002) dysfunction was prevented by treatment with a PARP inhibitor. Moreover, PARP-inhibition restored NAD(+), NADPH and ATP levels (Soriano et al., 2001a). Thus, PARP may deplete the intracellular concentration of its substrate NAD(+) thereby reducing the rate of glycolysis and ATP formation (Soriano et al., 2001b). The depletion in intracellular high energy phosphate levels, NAD(+) and NADPH by PARP activation may affect eNOS activity, since eNOS is an NADPH-dependent enzyme (Soriano et al., 2001a).

It can be argued that reduced NADPH diaphorase activity as found in our study may indicate dysfunction of the enzyme or the enzyme complex, possibly by alteration of the enzyme e.g. by advanced glycation end products, O-linked N-acetylglucosamine modification or damage by free radicals. Interestingly activity of NADPH diaphorase could be preserved by vitamin E treatment. Thus, endothelial dysfunction in hyperglycemia and its partial prevention by vitamin E may be related to enhanced inactivation of NO by free radicals or to alteration in intracellular enzymes as was shown for NADPH diaphorase. Another factor probably involved may be the intracellular energy crisis induced by oxidant-induced DNA breakage-dependent PARP activation as discussed above (Soriano et al., 2001a).

According to the literature reactive oxygen species overproduction is a key factor in hyperglycemia leading to reduced eNOS activity, advanced glycation end products, enhanced sorbitol formation, activation of protein kinase C, NFκB activation (Nishikawa et al., 2000) and inactivation of NO (Pieper et al., 1997; Stockklauser-Farber et al., 2000). Consequently, treatment with a radical scavenger could be a promising therapy. Accordingly, in our study vitamin E prevented from reduced basal NO release, which would support a role of reactive oxygen species either in inactivating NO or in alteration of eNOS. However, hyperglycemia-induced reduction in stimulated NO release was not influenced by vitamin E. This would contradict a general role of reactive oxygen species for altered eNOS leading to reduced

eNOS activity. Accordingly, Stockklauser-Farber et al. (2000) could show that eNOS activity was not altered in-vivo in diabetic rat hearts. This supports the hypothesis that in addition to the above mentioned mechanisms the signal transduction process is altered in hyperglycemia. In favor of this theory, we could show that the calcium signal following ATP stimulation of endothelial cells was significantly diminished in hyperglycemia (Salameh & Dhein, 1998).

*What are the reasons for the discrepant findings with vitamin E?*

There is some contradiction between the in-vivo study and the in-vitro results in our study which should be discussed: while in the in-vitro study vitamin E prevented from hyperglycemia-induced impairment of NO-release, we did not observe influence of vitamin E on LNNA-response. On the one hand there is a radical scavenging effect which might be present in both the in-vivo and the in-vitro situation. However, on the other hand the vasoprotective effect of α-tocopherol in-vivo also comprises binding of the vitamin to the vitamin E binding protein, transportation with the lipoproteins, and preservation of unsaturated bonds in essential fatty acids such as α-linolenic acid and eicosapentaenoic acid. Thus, in long-term in-vivo situation this more complex mechanism of action of vitamin E involving essential fatty acids might be affected in diabetes. Accordingly, a reduction in eicosapentaenoic acid/ arachidonic acid ratio was found in diabetic rats (Ikeda & Sugano, 1993). The metabolism in long-term diabetes probably is more complex than simple hyperglycemia as may be indicated by the loss of body weight in these rats. Moreover, this difference between results obtained from cultured cells and in-vivo results may indicate that diabetes mellitus means more than simply hyperglycemia. In addition, results from cultured porcine aortic endothelial cells can not completely simulate the situation in the long-term in-vivo mesentery artery of the diabetic rat. Another aspect is the duration of the disease or hyperglycemia. 7 months probably is in a rat model a long duration. Thus, positive effects seen with vitamin E in model with only 2 weeks or 2-3 months duration (Kunisaki et al., 1995; Karasu et al., 1997a, 1997b; Keegan et al., 1995) may be attenuated by the long duration of the disease.

To our surprise our study partially reflected the inhomogeneity of the literature (Skyrme-Jones et al., 2000; Nickander et al., 1994; Dagenais et al., 2001; Lonn, 2001, Lonn et al., 2001) with the in-vitro study demonstrating only a partial protective effect of vitamin E, while the in-vivo study only showed a negative effect of vitamin E deficiency but no effect of additional vitamin E. One factor jeopardizing the results with vitamin E in the literature is

that the „normal“ vitamin E supplementation is defined depending on the country: in USA vitamin E supplementation in „normal“ rat diet is approximately 30 mg/kg chow while in the United Kingdom 90-120 mg/kg chow is considered normal and in Germany 200 mg/kg chow (Lehr et al., 1999). To circumvent this problem in our study we investigated three different supplementations (10, 75, 1300 mg /kg chow). With regard to the recent findings of a lack of effect of vitamin E in the HOPE (Dagenais et al., 2001) and SECURE (Lonn et al., 2001) clinical studies, this emphasizes the necessity of preclinical in-vivo studies.

Regarding the comparison of the in-vivo and in-vitro studies, a first issue to mention is that endothelial cells grown on plastic or glass dishes are not identical to endothelial cells growing under in-vivo conditions exposed to flowing blood. A second issue is that cells in culture are considerably faster growing than cell in a blood vessel. This could mean additional stress to the cells and the metabolic activity of these growing cell in culture is probably higher than that of resting cells in a blood vessel. In line with this is the observation that vitamin E treatment exhibited positive preventive effects on cells additionally stressed by $H_2O_2$ (Asahina et al., 1995). Interestingly, in a study similar to ours a protective effect of vitamin E against coronary endothelial dysfunction in hearts of streptozotocin-diabetic rats has been described (Rösen et al., 1995). It could be argued that due to the higher oxygen consumption and energy metabolism in the heart as compared to mesenterium the formation of free radicals might be enhanced in diabetic high energy consuming tissue such as cardiac tissue. In support of this theory, a preventive vitamin E effect was observed in diabetic pregnant rats which also means a situation of enhanced oxidative stress (Kinalsky et al., 2000).

Our in-vitro study shows that vitamin E only prevented from reduced basal NO release but not from reduced stimulated NO-release. This might indicate disturbed signal transduction in hyperglycemia as was shown in a previous study investigating $Ca^{2+}$ signals in hyperglycemia (Salameh & Dhein, 1998). Moreover, the lack of effect on stimulated NO release might explain the inefficacy of vitamin E in the in-vivo model.

Thus, we conclude that high vitamin E treatment does not prevent from diabetic vascular dysfunction in this long-term rat model. However, a deficit in vitamin E seems deleterious for endothelial function in diabetes mellitus. Positive effects of vitamin E as seen in the cell culture model or by others in short term diabetic models (2 weeks to 3 months) may be attenuated with the long duration of diabetes.

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**Legends for figures and tables**

Fig. 1. Vascular reactions in non-diabetic and chronic (6 months) streptozotocin-induced diabetic (d.m.) rat mesenterial microvasculature with or without additional vitamin E treatment or with vitamin E deficiency. The figure shows the reactions of 4 branches of the mesenteric microcirculation (G1 to G4, initial diameters see table 1). All values are given as means±SEM of n=x experiments (for n see table 1). Statistical differences to non-diabetics is marked by an asterisk, statistical difference versus diabetes mellitus is indicated by a # ($p<0.05$).

Panel A shows the effect of 70 mM KCl on vascular tone in G1 to G4 mesenteric micovessels in % of initial diameter. Panel B gives the relaxation of the mesenteric micovessels in response to 1 µM glyceroyltrinitrate (GTN) as % of KCl contraction. In panel C the effect of 1 µM acetylcholine (ACh) is depicted as % of KCl contraction. Noote that in vitamine E deficient diabetics ACh no longer induced relaxation but resulted in a slight further contraction. Panel D shows the effect of 3 µM L-$N^G$-nitro-arginine on vascular tone. The contractions evoked by 3 µM L-$N^G$-nitro-arginine are normalized to the contractions elicited by KCl and data are given as % of KCl contraction.

Fig. 2. Difference spectrum of oxyhemoglobin versus methemoglobin obtained from cultered PAEC under normal (Panel A) and hyperglycemic (Panel B) conditions over a period of 90 minutes displaying the characteristic absorption maximum at 401 nm with an isobestic point at 411nm, a negative absorption maximum at 421 nm and straight lines between 401 and 411 nm. (± 1.25 nm tolerance regarding the precision of the instrument). The reaction was carried out in HEPES-buffered solution in the presence of 4.0 µ*M*/l Oxyhemoglobin at pH 7.4, 37°C as described (see methods section). Note the increase in Met-Hb formation evident from increasing absorption with time and its attenuation in hyperglycemic cells (panel B).

Fig. 3. Formation of nitric oxide in normo- and hyperglycemic endothelial cells with or without additional vitamin E treatment under basal conditions (left) and after stimulation with 1 mM ATP (right). All values are given as means±SEM of n=6 experiments. Statistical differences to non-diabetics is marked by an asterisk, statistical difference versus diabetes mellitus is indicated by a # ($p<0.05$). Note that vitamin E prevents from hyperglycemia-induced reduction in basal but not stimulated NO formation.

Fig. 4. Formation of nitric oxide in normo- and hyperglycemic (+15 mM D-glucose) endothelial cells and in cells treated with 15 mM L-glucose as osmotic control under basal conditions (left) and after stimulation with 1 mM ATP (right). All values are given as means±SEM of n=6 experiments. Statistical differences to non-diabetics is marked by an asterisk, statistical difference versus diabetes mellitus is indicated by a # ($p<0.05$). Note that 15 mM L-glucose (in contrast to 15 mM D-glucose) does not reduce NO formation.

Fig. 5 Original photographs showing NADPH diaphorase activity as assessed by histochemical nitro-blue tetrazolium staining in normoglycemic (upper panel) and hyperglycemic (lower panel) endothelial cells (400x magnification). Formation of the blue formazan stain indicates NADPH diaphorase activity. Note the typical positive dark staining in the perinuclear zone which is diminished in the hyperglycaemic cells.

Table 1

| | non-diabetic (n=9) | d.m. (n=6) | d.m. + Vit E (n=8) | d.m. - Vit E (n=6) |
|---|---|---|---|---|
| Bodyweight (g) | 418±8 | 228±10* | 237±3* | 199±7* |
| Blood glucose (mM) | 4.5±0.5 | 19±2* | 18.5±2* | 19.5±2* |
| Vitamin E plasma level (mg/l) | 10±1 | 10±2 | 19±2* | 2.2±1* |

Table 1: Bodyweight, blood glucose levels and vitamin E plasma levels of the four experimental groups given as means± SEM. Significant differences to the non-diabetic control series are indicated by an asterisk ($p<0.05$).

Table 2

| | non-diabetic (n=9) | d.m. (n=6) | d.m. + Vit E (n=8) | d.m. - Vit E (n=6) |
|---|---|---|---|---|
| G1 | 303±7 | 396±7* | 407±10* | 415±13* |
| G2 | 238±6 | 319±6* | 320±4* | 322±4* |
| G3 | 191±5 | 252±4* | 236±8* | 240±8* |
| G4 | 123±3 | 185±7* | 146±4*# | 160±8* |

Table 2: Initial diameter of mesenteric microvessels (generation G1 to G4) of non-daibetic control rats, diabetic rats and diabetic rats (d.m.) receiving high vitamin E diet (d.m. +Vitamin E) or receiving vitamin E deprived diet (d.m. - Vitamin E) under resting conditions given as means ± SEM. Significant differences to the non-diabetic control series are indicated by an asterisk ($p<0.05$). Significance versus diabetic animals is indicated by a # ($p<0.05$).

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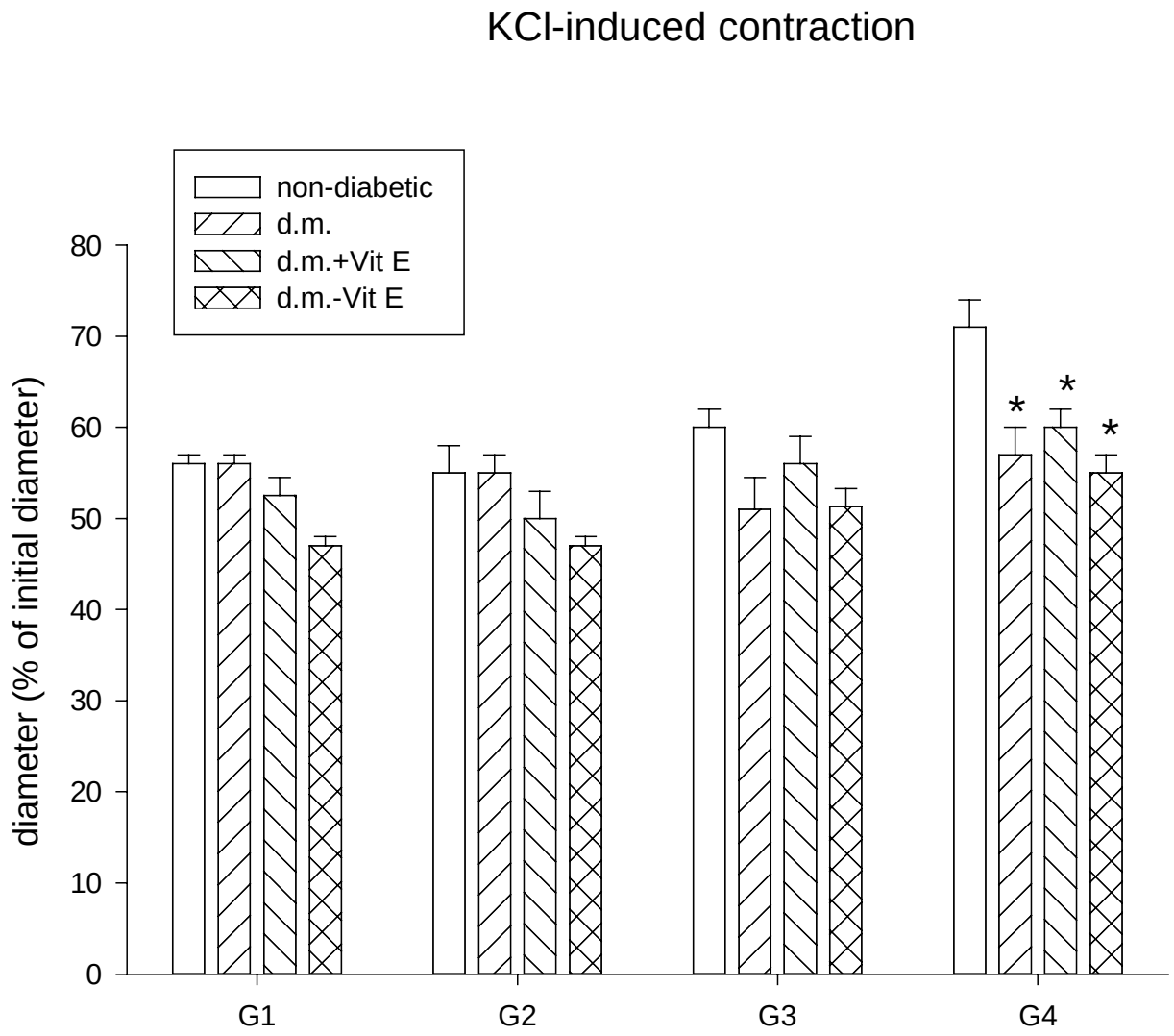




Fig. 1a

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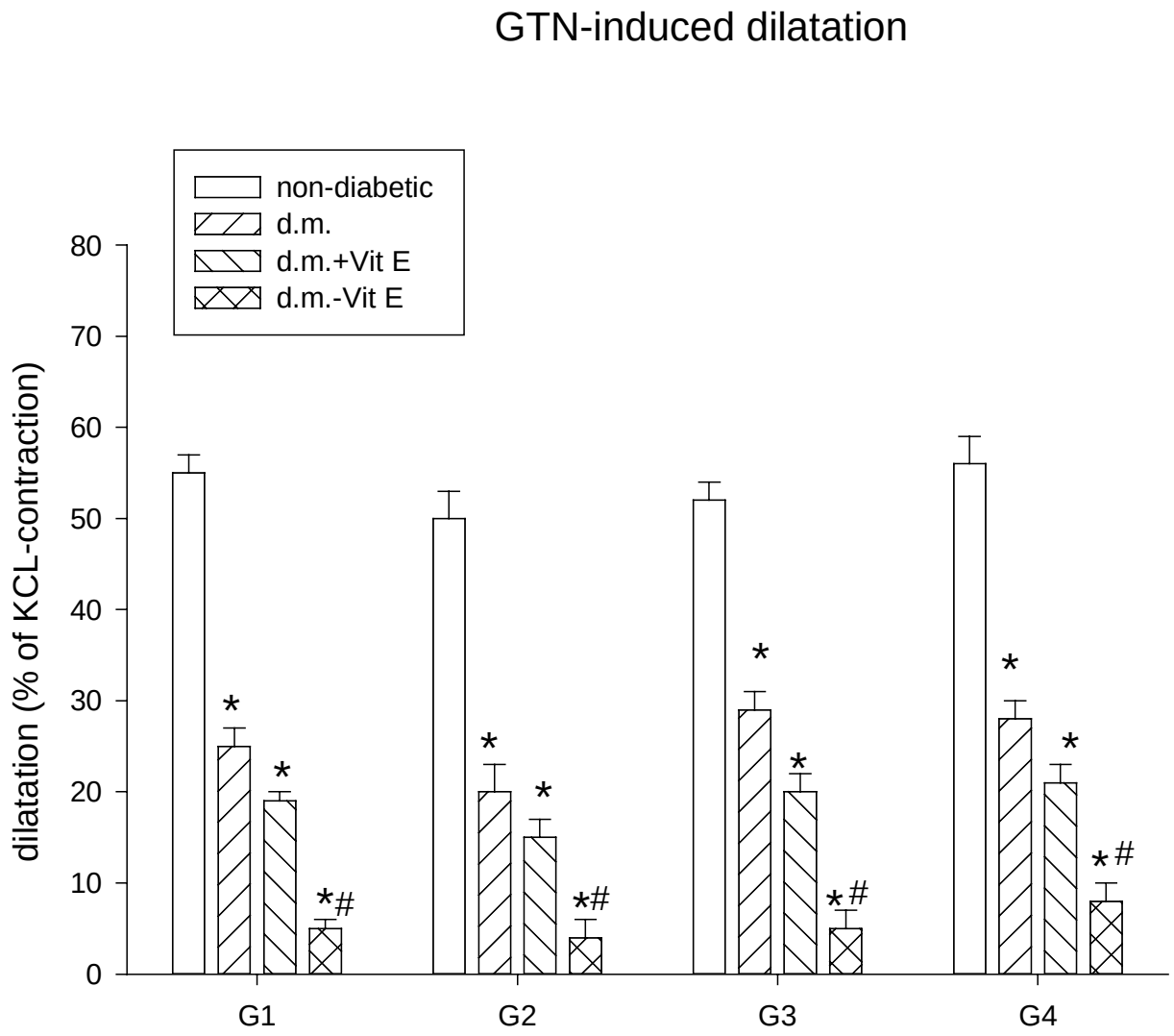




Fig. 1b

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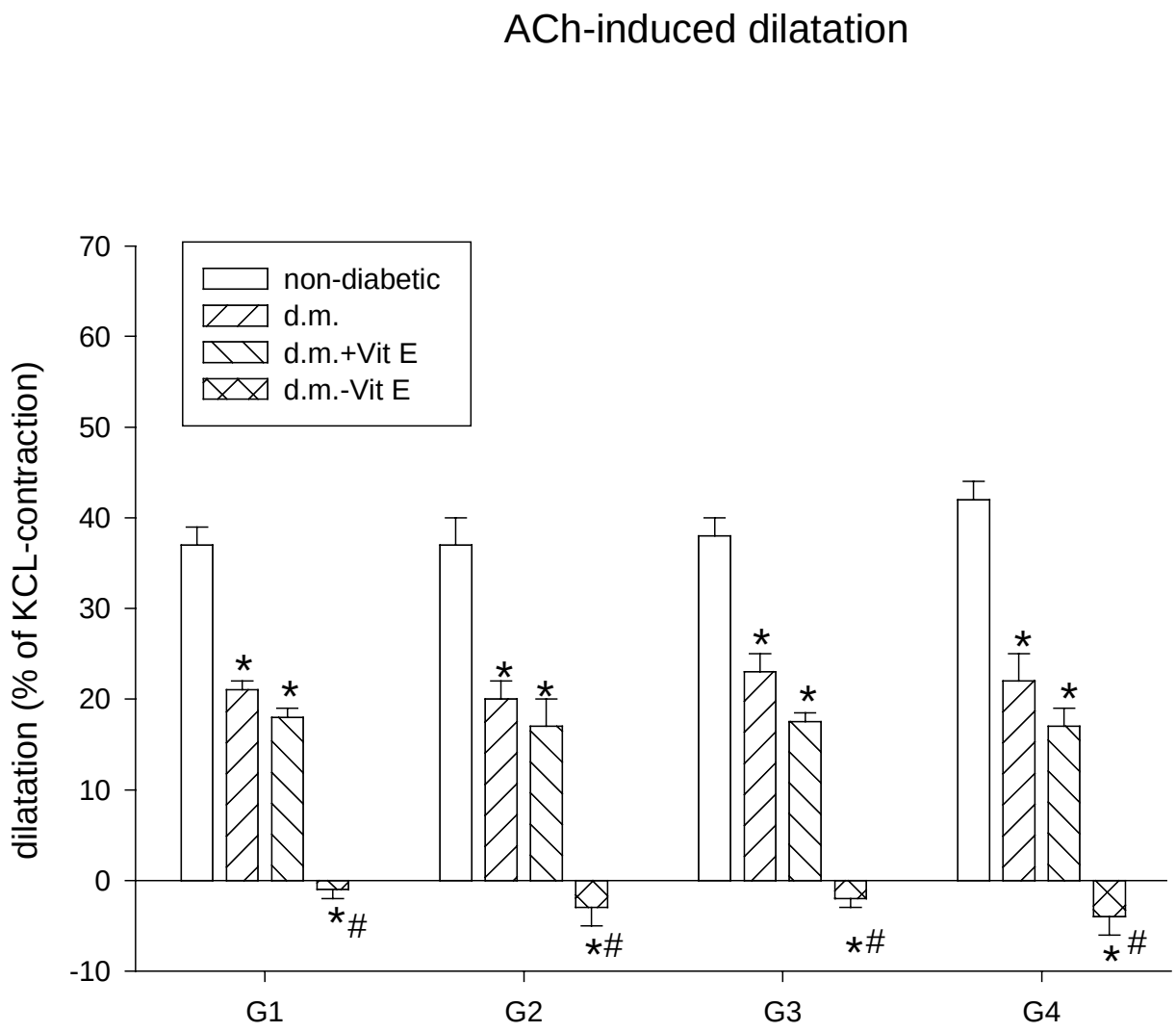


Fig. 1c

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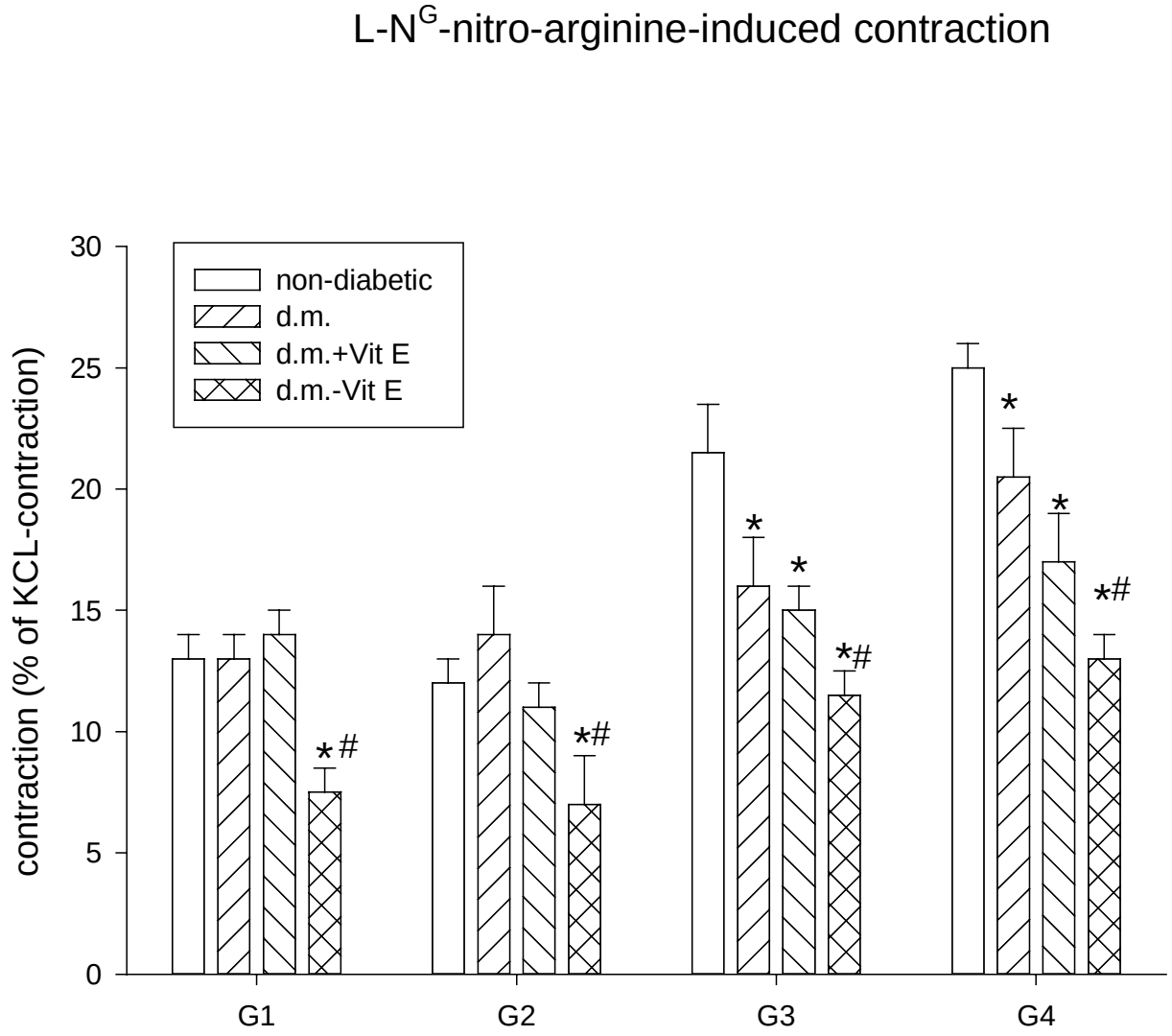




Fig. 1d

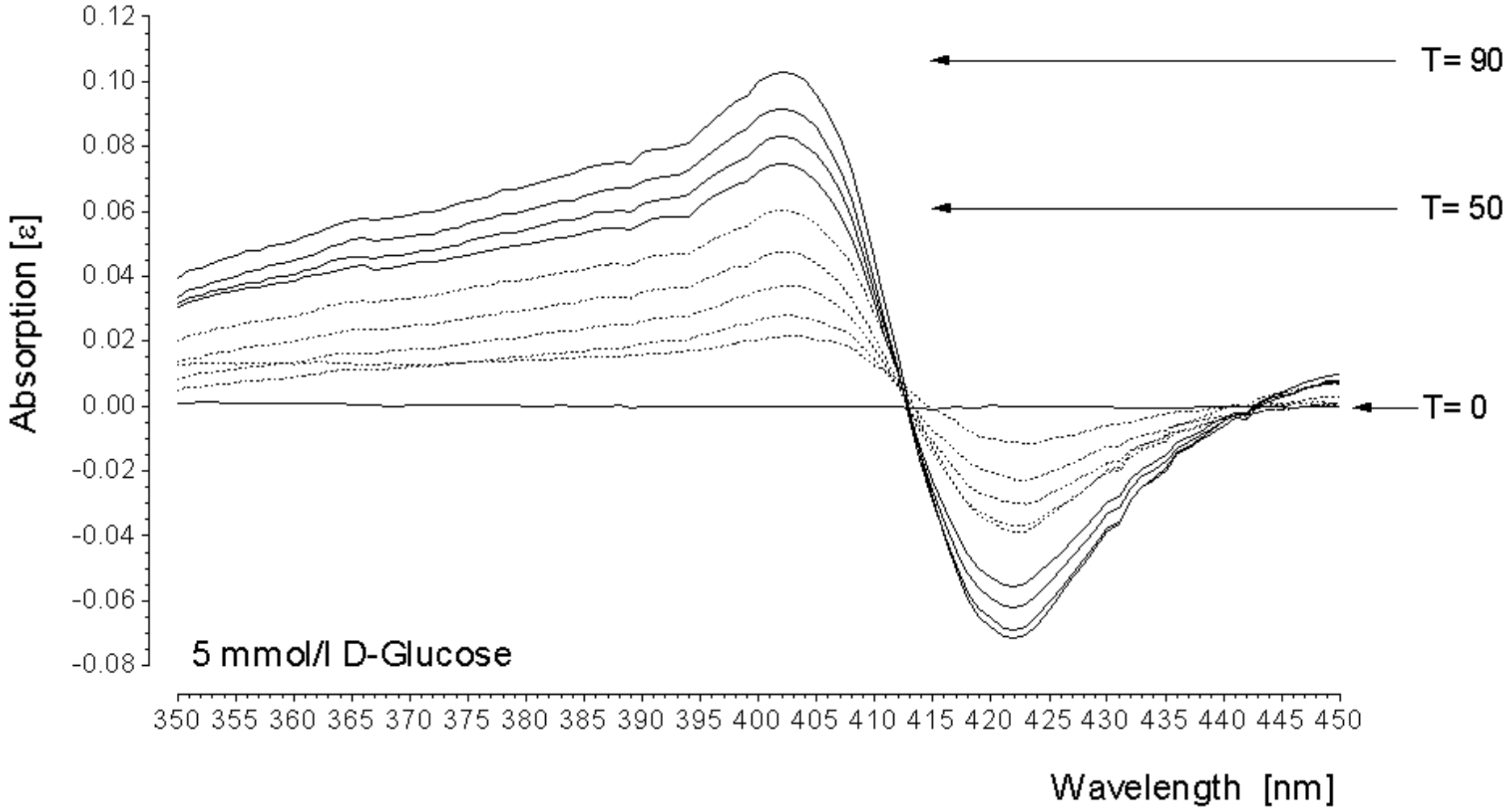


Fig. 2a

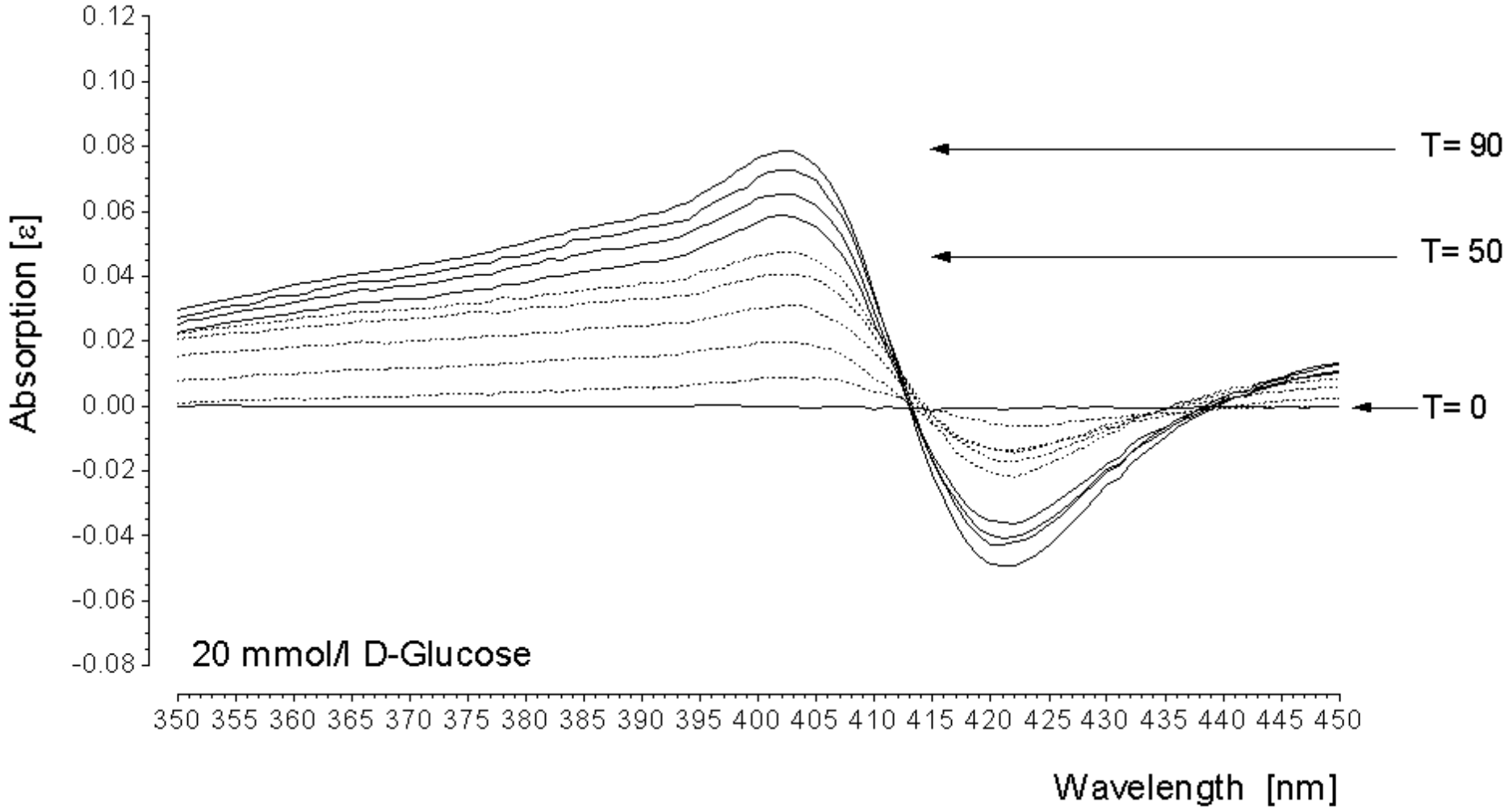


Fig. 2b

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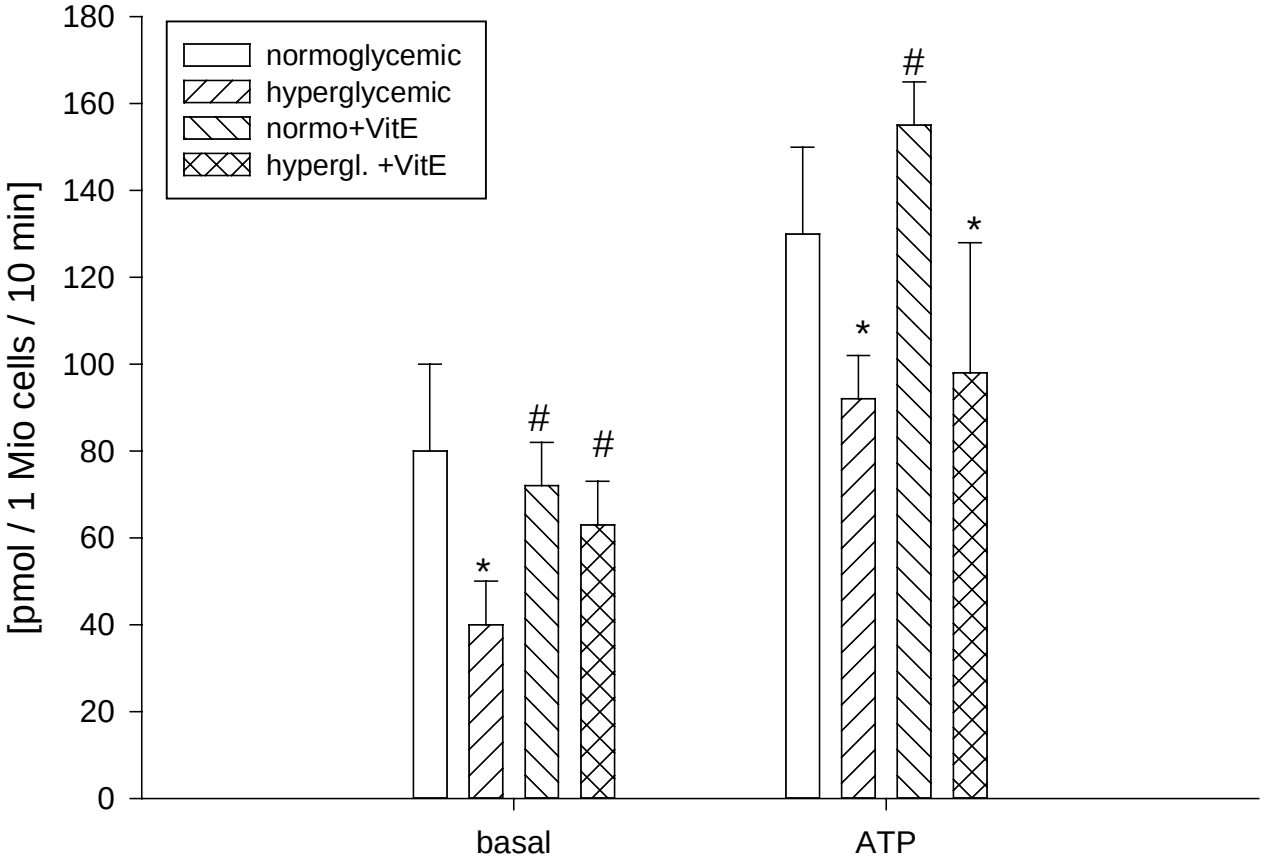


Fig. 3

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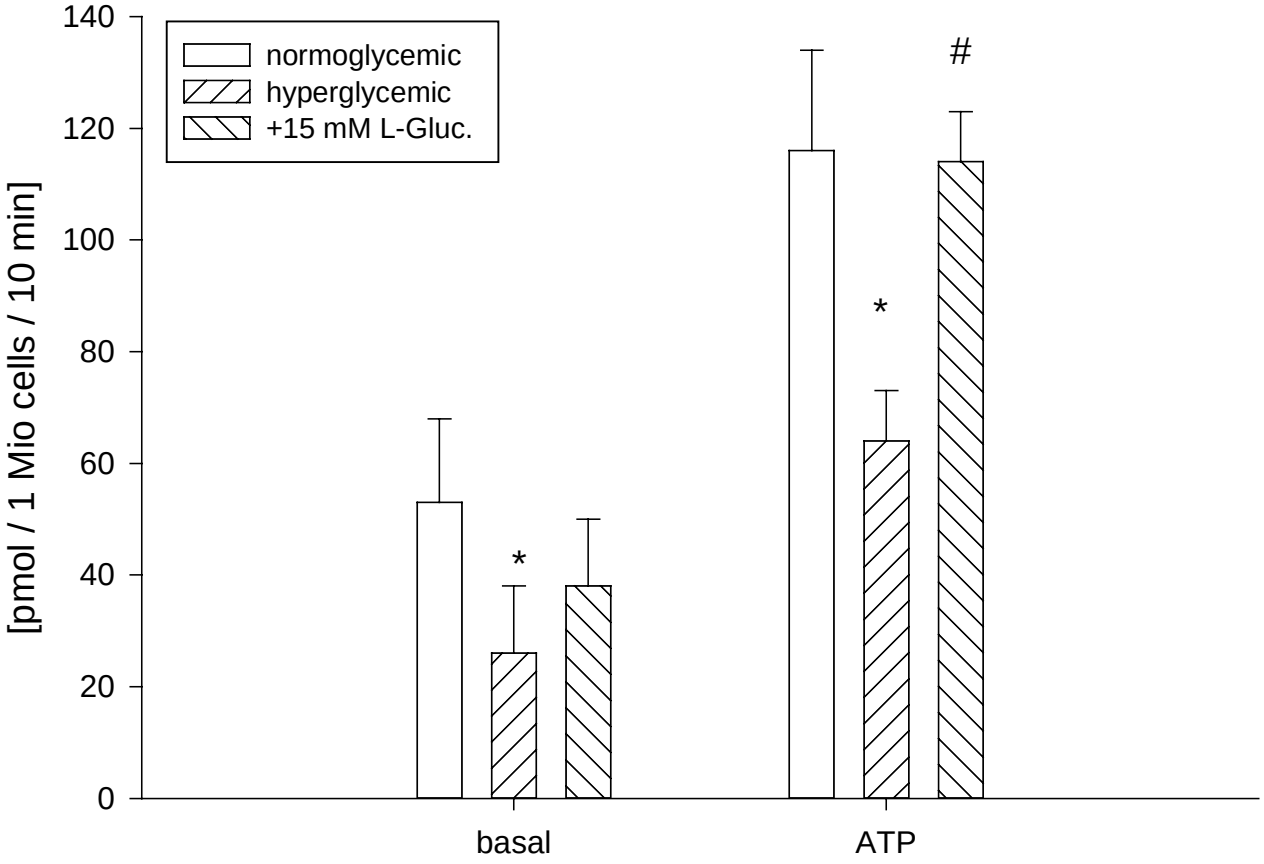


Fig. 4

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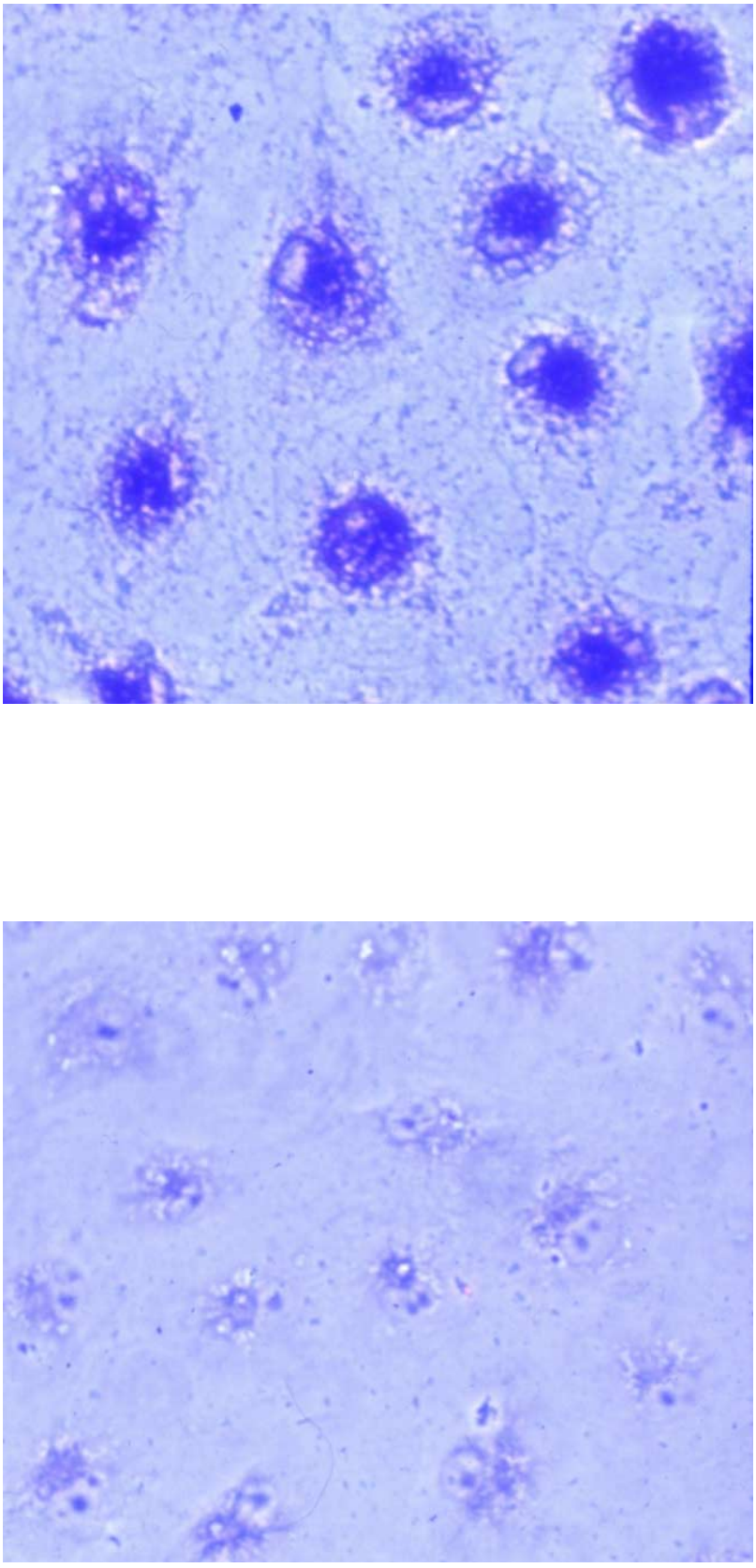

Fig. 5