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Effect of Hematoxylin on Glucose Metabolism in Soleus Muscle of Streptozotocin-Induced Diabetic Rats

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ABSTRACT—Hypoglycemic effect of hematoxylin was observed in streptozotocin-induced diabetic rats, of which plasma insulin levels were not affected. Investigation of hypoglycemic mechanism showed that hematoxylin stimulated glucose oxidation and glycogen synthesis in soleus muscle from diabetic rats in both basal and insulin stimulated state.

Keywords□Hematoxylin, Glucose oxidation, Glycogen synthesis, Streptozotocin diabetes

Diabetes mellitus is a complex syndrome characterized by hyperglycemia, secondary to deranged secretion and/or action of insulin; specific microvascular disease, i.e., accelerated atherosclerosis, complicated pregnancy, and increased tendency to infection. In addition, there is accelerated catabolism of both fat and protein. None of these findings is absolutely specific for diabetes, and it is clear that diabetes mellitus should not be considered simply as synonymous with hyperglycemia since there are many conditions in which there is impaired glucose tolerance which are not generally associated with the same spectrum of complications.

Insulin resistance, a characteristic feature of both insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM), is triggered at the level of the target tissue and can be induced by three general catagories: (a) an abnormal beta cell secretory product,[1] (b) circulating insulin antagonists,[2] or (c) a target tissue defect in insulin action.[3]

The overall scheme of insulin action represents a multistep sequences; the binding of insulin to receptors is only the initial event. A defect in any effector systems distal to receptor binding can be also lead to impaired insulin action and insulin resistance. These defect can involve abnormal coupling between insulin receptor complex and the glucose system.

In diabetic patients, an absolute or relative lack of insulin occures. So, many antidiabetic drugs were studied in the point of the relationship to insulin action for characterizing their mechanisms.[4-11]

The management of insulin resistance over recent years has not achieved. Although sulfonylureas[9] and biguanides[10,11] have been used in the treatment to NIDDM, the exact mechanism of the hypoglycemic action of these drug is still unclear and their side effects are too serious for either of these agents to be choice of drug.[12-14] Thus, exploration of new hypoglycemic agents which has confirmed effects and no toxicity, is necessary.

Hematoxylin, the active pigment component of *Hematoxylin campechianum* has widely been used as a staining meterial in cytologic research and a food additive such as a food coloring agent and an antioxidant,[15,16] and it increased definitely the respiratory activity of rat tumor of the spleen from cancerous animal in glucose-contained and glucose-free Ringer solution.[17]

In the present study we examined the effect of hemetoxylin on glucose metabolism in soleus muscles of streptozotocin-induced diabetic rats.

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Materials and Methods

Reagents—Streptozotocin, insulin, BSA, hyamine hydroxide, glycogen, and HBSS were purchased from Sigma Chemical Co., USA, (U-14C)-glucose from ICN Radioisotope Co., USA. All other chemicals were reagent grade.

Experimental Animals—Male sprague-Dawley rats were obtained from the Experimental Animal Breeding Center of Seoul National University and housed in a climate-controlled quarters maintained at 22± 1 ℃ of temperature, 60± 5% of humidity with a fixed 12 hour artificial light cycle (07 : 00 a.m. to 07 : 00 p.m.). They were allowed free access to water and a standard laboratory chaw (Samyang Feed Production Co.). The composition of the chaw is crude protein 22.1%, crude fat 3.5 %, calcium 0.6%. phosphorous 0.4%, crude cellulose 5.0%, and crude ash 8.0%.

After 2 weeks of acclimation, animals weighed between 170 and 200 gram were used as experimental animals.

Assay of Glucose Metabolism in Skeletal Muscle—Induction of diabetes mellitus and determination of plasma glucose and insulin level: Si) rats (170~200), all of them kept in identical condition, were fasted overnight and injected streptozotocin 40 mg/kg body weight through a tail vein. Streptozotocin was dissolved in citrated buffer (pH 4.0), kept in ice bath, and administered within 15~20 min. Rats with the blood glucose level of about 320 mg/dl were used as diabetic rats.

To determine the plasma glucose and insulin level, blood samples were collected from the retro-orbital plexus in non-fasting state in the moring (9 : 00~10 : 00 a.m.). Plasma glucose levels were determined using glucose oxidase kit (Beohringer Mannheim, GmbH, Mannheim, W. Germany). Plasma insulin levels were determined with radioimunoassay kit (Coat-A-count, Diagnostic Products Co., LA, USA).

Animal Grouping and Treatment—In the case of *in vivo* study, diabetic rats were into two equihyperglycemic groups. One group received hematoxylin (100 mg/kg/day, i.p. for 2 weeks), and the other served as an untreated diabetic control. Normal rats, which were treated with neither streptozotocin nor hematoxylin, were untreated normal control group.

Rats were killed by cervical dislocation. Hindlimbs were fixed and dissected out to expose soleus muscle as described by Chaudry *et al.* As intact soleus muscle of adult rat presents critical problems of substrate diffusion, a strip of soleus muscle has been used, which overcome this problem with practice, strips of soleus muscles weighing between 25 and 35 mg could regularly be obtained. Strips were let in iso-osmotic 0.9% NaCl solution at room temperature and were bletted and lightly atreched on stainless steel holder as described Cuendet *et al.*

Incubations of soleus muscles from and diabetic rats were carried out in a shaking incubator at 37 ℃, in 25 m*l* sample vial containing 3 m*l* of Krebs-Ringer bicarbonate-buffered (KRBB) medium, pH 7.4, with 10% fatty acid free bovine albumine and 2 mM pyruvate as energy source and with or without maximally effective concentration of insulin (0.05 U/m*l*). Each vial was sealed with rubber stopper and then gased with O_2/CO_2 (95 : 5 vol/vol) for 4 min. Actual incubations vere preceded by a 30 min preincubation, at the end of which vials and medium were changed and the preparations were gassed as described above. Actual incubations were carried out for in KRBB medium with 5 mM glucose (0.3 μCi (U-14C)-glucose/vial) and 1% defatted albumine, and with or without insulin (0.05 U/m*l*).

For the invatigation of *in vitro* effect of hematoxylin on soleus muscles from normal rats, various concentrations of hematoxylin (30, 3, 3.0 mg/*l*) were prepared in the incubation medium during the preincubation and the incubation periods, and other procedures were identical as described above.

Measurement of glucose oxidation: Oxidation of (U-14C)-glucose was measured by collecting 14 CO_2 produced by this substrate and counting it for radioactivity. The yield with this method (approximately 85%) was determined by the recovery of $NaHCO_3$ (25 nCi/m*l*) added to 3 m*l* of incubation medium.

At the end of the incubation, strips of filter paper placed to before the actual incubation in a hanging center well were moistened with 0.3 m*l* of 1 M hyamine hydroxide by injection and the incubation vials placed in an ice-cold water bath to 15 min. The vials were then opened briefly to remove and transfer the soleus muscle on its holder to −20 ℃. After resealing, the incubation medium was acidified with 0.5 m*l* 6 N H_2SO_4 and the CO_2, thus liberated, collected during 2 hour at 37 ℃. The center well with filter paper was transfered to the scintillation vial and assayed for radioactivity in a 10 m*l* toluene-based scintillation mixture.

Determination of Glycogen Synthesis: The rate of incorporation of glucose into glycogen was measured by 14C incorporation from (U-14C)-glucose into glycogen. Formation of glycogen from labeled glucose was measured after hydrolysis of the muscle with 0.5 m*l* 1 N NaOH at 80 ℃ in a heating block. Carrier glycogen (10 mg) was added to the hydrolate and glycogen was precipitated at −20 ℃ for 1 hour with 66% ethanol. The glycogen precipitate was obtained by centrifugation and was washed twice with 66% ethanol and then dissolved in 0.5 m*l* double distilled water. Its radioactivity was measured in 10 m*l* Bray's slution.

Statistical Analysis−All data were expressed as mean± S.D. Significance of the difference was examined by the Student's t-test for grouped samples.

Results and Discussion

The major insulin-dependent peripheral tissues are skeletal muscle, adipose tissue and liver, which represent approximately 45, 15 and 10% of body weight, respectively.[18,19] Indeed skeletal muscles *in vivo* are responsible for 35% glucose uptake in response to an intravenous glucose injection.[20] Thus, they are likely candidates to be involved in the overall insulin resistance in the rat. As there is actually no preparation of isolated myocyte sufficiently sensitive to insulin *in vitro* to enable one to construct a doseresponse curve, a muscle tissue was shpeen in this study. The soleus is a typical skeletal muscle that has high and constant activity, and consists of homogeneous fiber, and can be prepared intact.[21] There have been many reports that soleus muscle of adult rat presents critical problems of substrate diffusion. Thus, we have used a strip of soleus muscle preparation developed more recently which overcomes this problem.

Increased insulin binding in intact adipocyte,[22] hepatocyte[23] soleus muscle,[30] kidney,[24] and liver plasma preparation[25,26] has been demonstrated in the study using streptozotocin diabetic animals. Since streptozotocin destroy pancreatic β-cells and decreases insulin concentration,[27] increased insulin binding may reflect the lifting of a down-regulatory effect of normal insulin concentration.[28] Furthermore, previous studies have shown that prolonged insulinopenia, 3~4 week duration of diabetic state led to the decrease in both glucose transport and intracelluar glucose metabolism involving glycogen synthesis and lactate production, and resulted in post-receptor insulin resistance in skeletal muscles.[29]

Hematoxylin (100 mg/kg) treatment exhibited significant hypoglycemic effect, but was found to have no effect on plasma insulin levels (Table 1). This result suggests that hypoglycemic effect of hematoxylin might be mainly based on the stimulation of peripheral tissue. That is, the hypoglycemic action of hematoxylin does not result from an effect on insulin secretion, but rather from one or more extrapancreatic effects in streptozotocin-induced diabetic rats.

Comparing the basal glucose oxidation in soleus muscle from non-treated diabetic animals with that from treated subject. In the case of insulin-stimulated state, hematoxylin casused a significant increase (Fig. 1). As summarized in Fig. 2 hematoxylin significantly increased the basal and insulin-stimulated glycogen synthesis in muscles of streptozotocin-induced diabetic rats. These finding suggest that hematoxylin improve impaired insulin action in soleus muscle of streptozotocin-induced diabetic rat, and lead to hypothesis that hematoxylin may potentiate the insulin action or it may be able to cause insulin-like effect on glucose oxi-

Table 1. Characteristics of experimental animals

Group	Body Weight		Blood Glucose		Plasma Insulin	
	Initial	Final	Initial	Final	Initial	Final
NC	180± 5	275± 15	112± 9	115± 13	31± 2	30± 3
DC	189± 5	210± 3	325± 35	353± 47	22± 2	21± 4
DHT	180± 4	236± 16	348± 34[a)]	189± 24	21± 4	22± 4

100 mg/kg of hematoxylin was administered through i.p. for 2 weeks. Control group were adminstered the same volume of saline. Values are expressed as mean± SEM.
[a)] $p<0.05$ vs diabetic control group. Initial and Final represent the value before and after treatment of hematoxylin.
NC: normal control group, DC: diabetic control group, DHT: diabetic hematoxylin treated group.

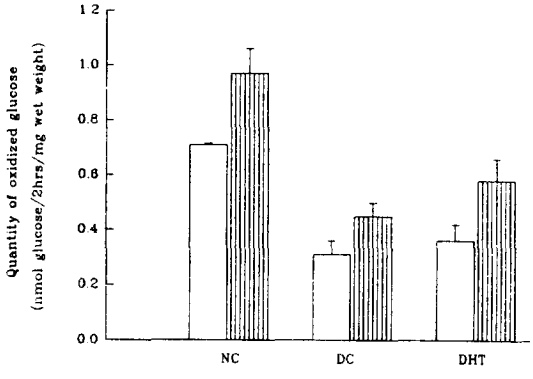

Fig. 1. ***In vivo*** **effect of hematoxylin on glucose oxidation.**
NC: normal control group, DC: diabetic control group, DHT: diabetic hematoxylin treated group.
□: basal state, ▥: insulin-stimulated state
[a)] $p<0.05$, vs. diabetic insulin-stimulated state.

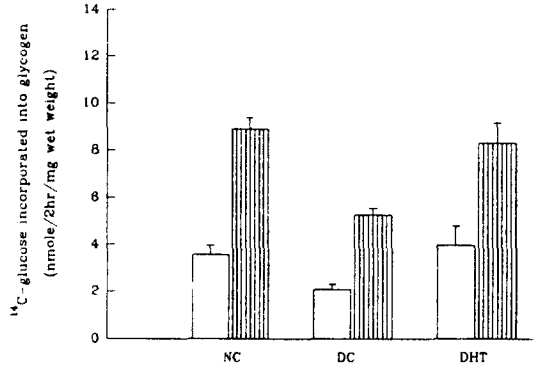

Fig. 2. ***In vivo*** **effect of hematoxylin on glucogen synthesis.**
NC: normal control group, DC: diabetic control group, DHT: diabetic hematoxylin treated group.
□: basal state, ▥: insulin-stimulated state
[a)] $p<0.05$, vs. diabetic insulin-stimulated state.

dation. But, since the overall increase in intracelluar glucose metabolism by the treatment with hematoxylin could be secondary to the effect on receptor binding or glucose transport and any further effect on intracellular glucose metabolism can not be precluded, the mechanism of these post-receptor action is still unkonwn. *In vitro* effects on glucose oxidation and glucose synthesis in soleus muscle were examined.

Exposure of rat soleus muscle to hematoxylin for two hour resulted in a significant increase in glucose oxidation and glycogen synthesis (Fig. 3, 4). In glucose oxidation assay hematoxylin had an effect only in the insulin-stimulated state; affected the glycogen synthesis both in basal and insulin-stimulated state. From the facts, the shorter treated time of hematoxylin in *in vitro* assay than that in *in vivo* assay, we could assume it exert its effect very rapidly in cells, and it might affect the path of glucose metabolism or that of insulin action mechanism.

We do not know whether it has insulin-like action or it has only affect the path of insulin action mechanism. It is, however, clear that the hypoglycemic action of hematoxylin is caused by extrapancreatic effect, such as the stimulation of glucose

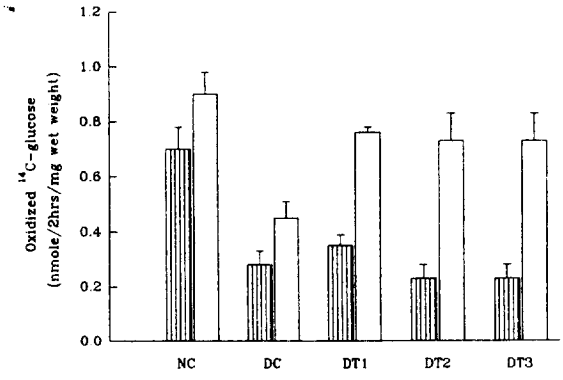

Fig. 3. *In vivo* effect of hematoxylin on glucose oxidation.
NC: normal control group, DC: diabetic control group, DT1: diabetic hematoxylin treated group (10^{-4} M), DT2: diabetic hematixylin treated group (10^{-5} M), DT3: diabetic hematoxylin treated group (10^{-6} M)
□: basal state, ▨ : insulin-stimulated state
[a] $p<0.05$, vs. diabetic insulin-stimulated state.

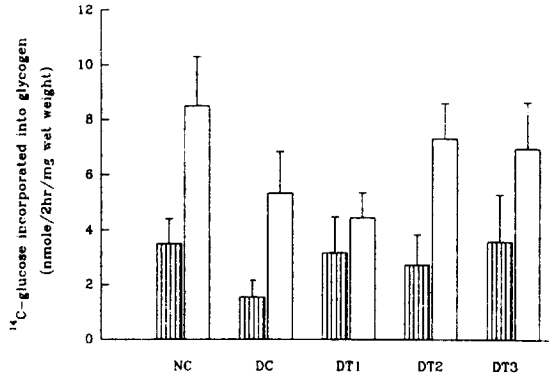

Fig. 4. *In vivo* effect of hematoxylin on glucogen synthesis.
NC: normal control group, DC: diabetic control group, DT1: diabetic hematoxylin treated group (10^{-4} M), DT2: diabetic hematixylin treated group (10^{-5} M), DT3: diabetic hematoxylin treated group (10^{-6} M)
□: basal state, ▨ : insulin-stimulated state
[a] $p<0.05$, vs. diabetic insulin-stimulated state.

metabolism in diabetic soleus muscle.

국문요약

천연 색소성분의 하나인 hematoxylin은 streptozotocin으로 유도한 당뇨병쥐에서 혈당강하 효과를 나타내는 것으로 관찰되었으며 이 때 혈중 insulin치에는 영향을 미치지 않았다. 혈당강하기전 연구의 일환으로 당뇨병쥐의 soleuo muscle에서의 포도당 대사에 미치는 영향을 검토한 결과 *in vivo* 및 *in vitro* 실험 모두에서 대사능이 증가되었고 이는 포도당대사 과정에서의 insulin 작용의 강화와 glycgen 합성의 증가에 기인하는 것으로 추정된다.

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