Insulin-Induced Hypoglycemia Stimulates Both Adrenaline and Noradrenaline Release from Adrenal Medulla in 21-Day-Old Rats

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ABSTRACT—It has been well-established that insulin-induced hypoglycemia evokes preferential adrenaline release from the adrenal medulla in fasted adult rats. The present study examined the responsiveness to hypoglycemia in fasted 21-day-old and 8-week-old rats. The recovery of adrenaline in the chromaffin granule fraction prepared from the 8-week-old rat adrenal homogenate decreased 30 min after subcutaneous injection of 3 U/kg insulin, whereas the recovery of both adrenaline and noradrenaline was diminished in 21-day-old rats. In electron microscopy, Ω -shaped profiles, indicative of exocytosis, were frequently observed in adrenaline- and noradrenaline-storing cells of 21-day-old rats. These results indicate that the responsiveness of the noradrenaline-storing cells to hypoglycemia in 21-day-old rats is different from that in young adult rats.

Keywords: Adrenal chromaffin cell, Hypoglycemia, Catecholamine release, Exocytosis, Infant rat

The importance of the sympathoadrenal system of fetuses and neonates has been emphasized for survival against various stresses such as hypoxia, asphyxia and hypoglycemia (1–3). It has been well-established that splanchnic nerves innervating the adrenal medulla are mainly responsible for the catecholamine release from adrenal chromaffin cells. In rats, the adrenal chromaffin cells are not yet innervated by splanchnic nerves at birth (4, 5). In 4-day-old rats, innervation of cholinergic nerves occurs (6). The number of innervations increases threefold during the first postnatal week (7). It has been reported that the function of the cholinergic nerves in the catecholamine biosynthesis and release in the chromaffin cells becomes fully mature in 10-day-old rats (4, 5). In the previous study, we demonstrated that nicotine hardly elicits catecholamine release from the adrenal medulla of 21-day-old rats (8). The responsiveness of the adrenal chromaffin cells of infant rats to various stimulations may be different from that in adult rats.

Insulin-induced hypoglycemia causes the preferential release of adrenaline from the adrenal medulla in various animals including rats (9–14). This release is blocked by transection of the splanchnic nerves (15). In the present study, the response of the adrenal chromaffin cells to hypoglycemia in fasted 21-day-old and 8-week-old rats was examined. Catecholamine release from the adrenal chromaffin cells was detected by biochemical methods

and observation of Ω -shaped profiles, indicative of exocytosis, using the electron microscope. Swelling of rough endoplasmic reticulum (rER) that indicates enhanced synthesis of secretory proteins, probably resulting in chromaffin granule regeneration, was also observed carefully. Insulin (3 U/kg) was injected subcutaneously. This is a dose that produces severe hypoglycemia in fasted adult rats 3 hr after the injection (14). As a result, hypoglycemia induced both adrenaline and noradrenaline release from the adrenal chromaffin cells of 21-day-old rats 30 min after the injection, whereas adrenaline release solely occurred in 8-week-old rats.

MATERIALS AND METHODS

Isolation of the adrenal chromaffin granule fraction

Male Wistar-Imamichi 21-day-old and 8-week-old rats (The Institute for Animal Reproduction, Ibaraki) were used after 1-week housing in our animal room for adaptation. The animals were kept under lighting conditions of 12L–12D, at $24 \pm 2^\circ\text{C}$, and they had free access to food and water. They were fasted from 16:00 pm to the next morning at 10:00 am; Then, 3 U/kg insulin, dissolved in saline, was injected subcutaneously. The animals were killed by decapitation 30 min or 3 hr after the injection. Adrenals were dissected immediately from the animals and homogenized with a Teflon pestle homogenizer in a

medium composed of 0.3 M sucrose, 20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 5.9), 2 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The chromaffin granule fraction was prepared from a mixture of equal volumes of the homogenates obtained from 6 rats (10 mg protein in total) by linear sucrose gradient centrifugation, as reported previously (16). All procedures described above were carried out at 4°C.

Electron microscopy

Adrenal glands were excised after decapitation as soon as possible. Segments of the adrenal medulla were processed for electron microscopic study as previously reported (8).

Determination of catecholamines, serum glucose and protein concentration

Catecholamines were analyzed by HPLC as reported previously (16). Serum glucose concentration was determined by the enzymatical method using a commercially available kit (F-kit glucose; Boehringer Mannheim, Germany). Protein concentration was measured by the method of Bradford (17) with bovine γ -globulin as the standard.

Chemicals

EGTA, MES and PMSF were purchased from Sigma Chem., Co., St. Louis, MO, USA. The dye reagent for protein determination and γ -globulin were from Bio-Rad, Richmond, CA, USA. Pig insulin was the product of Yamanouchi Pharmaceutical Co., Tokyo. Glutaraldehyde, paraformaldehyde and OsO₄ were from TAAB Laboratories Equipment, Ltd., Berks, England. Other chemicals were all analytical or electron microscopic grade.

Statistical analyses

Data are expressed as means $\pm$ S.E. To define statistically significant differences in the catecholamine concentration between the control and insulin-treated rats, the data were analyzed by Student's *t*-test.

RESULTS

Effect of hypoglycemia on adrenal chromaffin cells in 8-week-old rats

Although the behavioral activity of fasted 8-week-old rats seemed to become somewhat low 30 min after subcutaneous injection of 3 U/kg insulin, convulsion was not evoked. The animals were killed by cervical dislocation 30 min after the injection. As shown in Table 1, serum glucose concentration was decreased significantly by insulin treatment. Liver weight also decreased markedly.

Table 1. Effect of hypoglycemia on adrenal catecholamine concentration in 8-week-old rats

	Control	Insulin
n	6	6
Body wt. (g)	262 $\pm$ 5	233 $\pm$ 3
Liver wt. (g/100 g BW)	5.03 $\pm$ 0.12	3.45 $\pm$ 0.06**
Serum glucose (g/l)	1.21 $\pm$ 0.02	0.14 $\pm$ 0.02**
Adrenal wt. (mg/100 g BW)	15.8 $\pm$ 0.6	17.7 $\pm$ 1.4
Catecholamine concentration (nmol/mg protein)		
Adrenaline	18.5 $\pm$ 1.5	15.0 $\pm$ 1.1
Noradrenaline	2.41 $\pm$ 0.21	2.17 $\pm$ 0.24

**P < 0.005 vs control.

Fig. 1. Separation of the adrenal chromaffin granule fraction in 8-week-old rats 30 min after insulin injection by linear sucrose gradient centrifugation. The 10,000 $\times$ g precipitate fraction, prepared from a mixture of equal volumes of the adrenal homogenates obtained from 6 rats (10 mg protein), was centrifuged on a linear concentration gradient of sucrose from 0.8 to 1.6 M, and fractions of 0.25 ml were taken. The figure shows typical data from two separate experiments. ○: Control, ●: Insulin.

Fig. 2. Electron micrographs of adrenaline-storing cells in 8-week-old rats. Panel a: control, Panel b: 30 min after 3 U/kg insulin injection. A: adrenaline-storing cell, E: endothelial cell, F: fibroblast, p: perivascular space. Note that Ω -shaped profiles are present in an adrenaline storing cell of an insulin-treated animal (arrowheads in panel b). Arrows in panel b indicate chromaffin granules, probably just before exocytosis. Bars indicate 1 μ m.

The adrenaline and noradrenaline concentration of the adrenal homogenate tended to decrease, but this was not statistically significant. As indicated in Fig. 1 (a and b), the adrenaline- and noradrenaline-storing chromaffin granule fractions were obtained by linear sucrose gradient centrifugation in fraction numbers 2 to 6. The recovery of adrenaline from 10 mg protein of the adrenal homogenate in the chromaffin granule fraction was decreased in insulin-treated animals (Fig. 1a). The recovery of noradrenaline was scarcely changed by the treatment (Fig. 1b).

Figure 2a shows an electron micrograph of an adrenaline-storing chromaffin cell of a 8-week-old control rat. In the control, Ω -shaped profiles, indicative of exocytosis, were rarely found. In the adrenaline-storing cells of insulin-treated animals, Ω -shaped profiles were

frequently observed (Fig. 2b, arrowheads). Arrows in Fig. 2b indicate the chromaffin granules probably just before exocytosis. In the noradrenaline-storing cells of control and insulin-treated rats, Ω -shaped profiles were hardly found (data not shown).

Three hours after insulin injection, severe convulsion was evoked in almost all rats. In these animals, preferential decrease of the recovery of adrenaline in the chromaffin granule fraction occurred (Fig. 3 a and b). In electron microscopy, the number of chromaffin granules was decreased markedly, and a remarkable number of vacuoles were observed in the adrenaline-storing chromaffin cells, but there were hardly any morphological changes in the noradrenaline-storing cells (data not shown).

In the cell membrane of the synapse buttons of cholinergic nerve fibers, innervated to the noradrenaline-

storing cells of insulin-treated animals, Ω -shaped profiles formed by acetylcholine release were not present at 30 min and 3 hr after the injection (data not shown).

Effect of hypoglycemia on adrenal chromaffin cells in 21-day-old rats

The behavioral activity of insulin-treated 21-day-old rats was almost the same as that in the control animals 30 min after the injection. As indicated in Table 2, the serum glucose concentration of 21-day-old fasted rats was significantly reduced 30 min after the insulin injection. In these animals, liver weight also decreased markedly. Both the adrenaline and noradrenaline concentration in the adrenal homogenate was decreased significantly by insulin treatment ($P < 0.005$ and $P < 0.05$ vs control, respectively). As indicated in Fig. 4 (a and b), the adrenaline-

Table 2. Effect of hypoglycemia on adrenal catecholamine concentration in 21-day-old rats

	Control	Insulin
n	7	7
Body wt. (g)	25.8 ± 0.4	25.8 ± 0.4
Liver wt. (g/100 g BW)	4.29 ± 0.06	$3.04 \pm 0.04^{**}$
Serum glucose (g/l)	1.24 ± 0.12	$0.16 \pm 0.02^{**}$
Adrenal wt. (mg/100 g BW)	27.0 ± 0.7	$29.5 \pm 0.3^{**}$
Catecholamine concentration (nmol/mg protein)		
Adrenaline	11.9 ± 0.8	$9.0 \pm 0.2^{**}$
Noradrenaline	2.42 ± 0.15	$2.01 \pm 0.09^*$

* $P < 0.05$, ** $P < 0.005$ vs control.

Fig. 3. Separation of the adrenal chromaffin granule fraction in 8-week-old rats 3 hr after insulin injection by linear sucrose gradient centrifugation. The $10,000 \times g$ precipitate fraction, prepared from a mixture of equal volumes of the adrenal homogenates obtained from 6 rats (10 mg protein), was centrifuged on a linear concentration gradient of sucrose from 0.8 to 1.6 M, and fractions of 0.25 ml were taken. This experiment was performed one time. ○: Control, ●: Insulin.

Fig. 4. Separation of the adrenal chromaffin granule fraction in 21-day-old rats 30 min after insulin injection by linear sucrose gradient centrifugation. The $10,000 \times g$ precipitate fraction, prepared from a mixture of equal volumes of the adrenal homogenates obtained from 7 rats (10 mg protein), was centrifuged on a linear concentration gradient of sucrose from 0.8 to 1.6 M, and fractions of 0.25 ml were taken. The figure shows typical data from two separate experiments. ○: Control, ●: Insulin.

Fig. 5. Electron micrograph of 21-day-old control rat adrenaline- and noradrenaline-storing chromaffin cells. A: adrenaline-storing cell, NA: noradrenaline-storing cell, n: synapse button of preganglionic cholinergic nerve, p: perivascular space. Note that an Ω -shaped profile is present in the adrenaline-storing cell (large arrowhead). An arrow indicates an Ω -shaped profile found in the cell membrane of a synapse button facing the synaptic cleft. Small arrowheads indicate acetylcholine-storing secretory granules. The bar indicates 1 μ m.

and noradrenaline-storing chromaffin granule fractions were obtained by sucrose gradient centrifugation in fraction numbers 3 to 7. The adrenaline and noradrenaline contents in the chromaffin granule fraction were also depleted markedly by the injection.

As shown in Fig. 5, adrenaline- and noradrenaline-storing chromaffin cells in 21-day-old control rats are morphologically very similar to those in the 8-week-old control animals (Fig. 2a). As indicated with a large arrowhead in the figure, an Ω -shaped profile is found, although this is a rare case. In this photograph, a synapse button of a cholinergic nerve innervated to the

noradrenaline-storing cell is present (n). Several very small acetylcholine-storing secretory granules, that are less electron dense than the adrenaline-storing chromaffin granules, exist in the synapse button (small arrowheads). An Ω -shaped profile formed by acetylcholine release is observed in the cell membrane of the synapse button facing the synaptic cleft (arrow).

Figure 6 shows an electron micrograph of adrenaline-storing and noradrenaline-storing cells of an insulin-treated animal. In both cells, Ω -shaped profiles are frequently observed (arrowheads). Sometimes large Ω -shaped profiles are present (arrow), probably produced by the fusion of several chromaffin granules that had occurred just before exocytosis.

As indicated in Fig. 7, very large size granules were sometimes observed in the noradrenaline-storing cells of 21-day-old insulin-treated rats (arrowheads). In these granules, an electron-dense core was present. Swelling of the rER was also found in these cells (arrows).

DISCUSSION

In the present study, Ω -shaped profiles were found in the cell membrane of synapse buttons of cholinergic nerve terminals innervating the adrenal medulla in 21-day-old rats. This indicates that acetylcholine release from the nerve terminals occurs to some extent in 21-day-old rats. The cholinergic nerves are at least functionally active, although the degree of maturity is not clear from the present data.

In the present study, serum glucose levels of 21-day-old and 8-week-old rats were remarkably decreased 30 min after insulin injection. No convulsions were evoked by hypoglycemia in these animals. It has been reported that excitatory amino acids such as aspartate and glutamate are increased in synaptic clefts of the central nervous system by hypoglycemia, resulting from a decrease in cellular uptake due to energy depletion, and that these amino acids are neurotoxic (18). These increased excitatory amino acids in synaptic clefts of the central nervous system probably result in severe convulsion and catecholamine output from the adrenal medulla. The decrease of the recovery of catecholamines in the chromaffin granule fraction observed 30 min after insulin treatment in 21-day-old and 8-week-old rats is probably not induced by the effect of aspartate and glutamate in the central nervous system, because no convulsions were evoked by hypoglycemia in these animals.

In the present study, not only adrenaline but also noradrenaline was released from the adrenal medulla by insulin treatment in 21-day-old rats, whereas preferential adrenaline release occurred in 8-week-old rats. The present results indicate that the responsiveness of the

Fig. 6. Electron micrograph of 21-day-old insulin-treated rat adrenaline- and noradrenaline-storing chromaffin cells. Animals were killed 30 min after insulin injection. A: adrenaline-storing cell, NA: noradrenaline-storing cell, E: endothelial cell, F: fibroblast, p: perivascular space. Note that several Ω -shaped profiles are present in both the adrenaline- and noradrenaline-storing cells (arrowheads). The arrow indicates a large Ω -shaped profile, probably produced by fusion of several chromaffin granules that occurred just before exocytosis. The bar indicates 1 μ m.

noradrenaline-storing cells of infant rats against hypoglycemia is somewhat different from that in young adult rats. It is well-known that the serum glucose level is elevated more effectively by adrenaline than noradrenaline and that noradrenaline is a more potent vasopressor when compared to adrenaline. The merit of the noradrenaline release in the protection against hypoglycemia for the survival of infant rats is unknown at present.

In the previous study, the catecholamine concentration of the adrenals in 8-week-old rats was not significantly changed by 5 mg/kg nicotine stimulation, whereas the contents in the chromaffin granule fraction decreased markedly (8). The data of the previous report suggest that the chromaffin granules recovered in the chromaffin granule fraction are mature ones and that these are mainly linked to catecholamine secretion. The recovery of adrenaline in this fraction from the homogenate of adrenal medulla in control animals estimated in the previous study was about 66% (8). The other portion of the granules is probably not involved in catecholamine

release. Therefore, the magnitude of the decrease in this fraction is more apparent than that in the homogenate. In the present study, insulin did not significantly reduce the adrenaline concentration of the adrenals in 8-week-old rats, while the recovery in the chromaffin granule fraction was remarkably decreased by the administration. This is probably because insulin-treatment only induces exocytosis in the mature chromaffin granules.

In the present study, large granules were occasionally found in noradrenaline-storing cells of insulin-treated 21-day-old animals. These granules had an electron-dense core. The core has been observed in noradrenaline-storing chromaffin granules, which were pre-fixed by glutaraldehyde and post-fixed by OsO_4 and then stained with uranyl acetate and lead citrate (19). Therefore, the granules seen in the present study were probably not vacuoles but chromaffin granules. Fusion of several chromaffin granules at high Ca^{2+} concentration (about 1 μM) has been reported in *in vitro* studies (20, 21). These large chromaffin granules, observed in the present study, may be produced by a

Fig. 7. Electron micrograph of a noradrenaline-storing cell in a 21-day-old insulin-treated rat. Animals were killed 30 min after insulin injection. NA: noradrenaline-storing cell, E: endothelial cell, p: perivascular space. Note that very large granules that have an electron-dense core are present (arrowheads) and that swelling of the rER is observed (arrows). The bar indicates 1 μ m.

high cytosolic Ca^{2+} concentration, resulting from Ca^{2+} influx caused by acetylcholine release from cholinergic nerve terminals innervating the noradrenaline-storing cells. In these cells, swelling of the rER was simultaneously found. This indicates that synthesis of secretory proteins such as chromogranin A is enhanced by insulin-treatment, probably resulting in chromaffin granule regeneration.

REFERENCES

- 1 Lagercrantz H: Catecholamine surge at birth in the human infant. *In* Catecholamines: Basic and Peripheral Mechanisms, Edited by Usdin R, pp 113–120, Alan R Liss Inc, New York (1984)
- 2 Jones CT and Lagercrantz H: Catecholamines in perinatal medicine: introduction. *In* Catecholamines: Basic and Peripheral Mechanisms, Edited by Usdin R, pp 111–112, Alan R Liss Inc, New York (1984)
- 3 Slotkin TA and Seidler FJ: Role of adrenomedullary catecholamine release in survival during neonatal hypoxia. *In* Catecholamines: Basic and Peripheral Mechanisms, Edited by Usdin R, pp 129–131, Alan R Liss Inc, New York (1984)
- 4 Slotkin TA, Smith PG, Lau C and Bareis DL: Functional aspects of development of catecholamine biosynthesis and release in the sympathetic nervous system. *In* Biogenic Amines in Development, Edited by Parvez H and Parvez S, pp 29–48, Elsevier, Amsterdam (1980)
- 5 Slotkin TA, Chantry CJ and Bartolome J: Development of central control of adrenal catecholamine biosynthesis and release. *Adv Biosci* 36, 95–102 (1982)
- 6 Tomlinson A and Coupland RE: The innervation of the adrenal gland. IV. Innervation of the rat adrenal medulla from birth to old age. A descriptive and quantitative morphometric and biochemical study of the innervation of chromaffin cells and adrenal medullary neurons in Wistar rats. *J Anat* 169, 209–236 (1990)
- 7 Dalnock GKV and Menssen HD: A quantitative electron microscopic study of the effect of glucocorticoids in vivo on the early postnatal development of paraneuronal cells in the carotid body and the adrenal medulla of the rat. *Anat Embryol* 174, 307–319 (1986)
- 8 Fujino Y and Fujii T: Lack of the response to nicotine in 21-day-old rat adrenal chromaffin cells in vivo. *J Biochem* 116, 967–972 (1994)
- 9 Cannon WB, McIver A and Bliss SW: Studies on the conditions of activity in endocrine glands. XIII. A sympathetic and adrenal mechanism for mobilizing sugar in hypoglycemia. *Am J Physiol*

- 69, 46–66 (1924)
- 10 Dunner H: The influence of the blood glucose level on the secretion of adrenaline and noradrenaline from the supra-renal. *Acta Physiol Scand* **28**, Supp **102** (1953)
- 11 Coupland RE: The effects of insulin, reserpine and choline 2:6-xylylether bromide on the adrenal medulla and medullary autografts in the rat. *J Endocrinol* **17**, 191–196 (1958)
- 12 Gagner JP, Gauthier S and Sourkes TL: Descending spinal pathways mediating the responses of adrenal tyrosine hydroxylase and catecholamines to insulin and 2-deoxyglucose. *Brain Res* **325**, 187–197 (1985)
- 13 Carbonaro D, Mitchell JP, Hall FL and Bulliet PR: Altered reactivity of the rat adrenal medulla. *Brain Res Bull* **21**, 451–458 (1988)
- 14 Vollmer RR, Baruchin A, Kolibal-Pegher SS, Corey SP, Stricker EM and Kaplan BB: Selective activation of norepinephrine- and epinephrine-secreting chromaffin cells in rat adrenal medulla. *Am J Physiol* **263**, R716–R721 (1992)
- 15 Hokfelt B: Noradrenaline and adrenaline in mammalian tissues. *Acta Physiol Scand* **25**, Supp **92**, 1–134 (1951)
- 16 Fujino Y, Fujii T and Daimon T: Presence of laminin B chain-like protein in the bovine and rat adrenal chromaffin granules. *J Biochem* **115**, 615–621 (1994)
- 17 Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254 (1976)
- 18 Sandberg M, Butcher SP and Hagberg H: Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: In vivo dialysis of the rat hippocampus. *J Neurochem* **47**, 178–184 (1986)
- 19 Coupland RE, Pyper AS and Hopwood D: A method for differentiating between noradrenaline- and adrenaline-storing cells in the light and electron microscope. *Nature* **201**, 1240–1242 (1964)
- 20 Ekerdt R, Dahl G and Gratzl M: Membrane fusion of secretory vesicles and liposomes: two different type of fusion. *Biochim Biophys Acta* **646**, 10–22 (1981)
- 21 Creutz CE and Pollard HB: A cell-free model for protein-lipid interaction in exocytosis: aggregation and fusion of chromaffin granules in the presence of calcium, synexin and *cis*-unsaturated fatty acids. *Biophys J* **37**, 119–120 (1982)