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Bioorganic & Medicinal Chemistry Letters 14 (2004) 481–483
Bioorganic & Medicinal Chemistry Letters

Enzymatic and hyperglycemia stability of chemically modified insulins with hydrophobic acyl groups

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Received 20 September 2003; accepted 20 October 2003

Abstract—The acylated insulins were synthesized by the reaction of insulin protected by *p*-methoxybenzoxy carbonyl azide at Gly-A1 site with *N*-hydroxysuccinimide ester of caproic acid or benzoic acid (Cap-insulin and Bz-insulin). The noteworthy aspects are as follows: (a) the acylated insulins were more stable to the decomposition by various digestive enzymes as compared with native insulin in vitro. (b) Animal experiments using normal rats in vivo revealed that the Bz-insulin had an effective hypoglycemia activity almost similar to that of native insulin.

In insulin dependent diabetes mellitus (IDDM), the continual administration of insulin is indispensable to the patients for the prevention of some serious complications.[1] Although oral administration is one method for an appropriate dosage with the smallest burden to the patients, this treatment has not yet been successful because insulin is a hydrophilic peptide hormone which has high degradability by the proteolytic enzymes in the digestive tract and low permeability to the mucous membrane of intestines.[2]

Hybrid liposomes, first developed by Ueoka et al., can be prepared by simply ultrasonicating a mixture of vesicular and micellar molecules in the buffer solution. Shape, size, and the temperature of phase transition of these liposomes can be controlled by changing the constituents and compositional ratios.[3] Hybrid liposomes are highly expected to be very useful as a new material in the medical field because of their bioadaptability, easiness of preparation than the ordinary methods, and no contamination with any organic solvents. It has been reported that hybrid liposomes demonstrated remarkable inhibitory effects on the growth of lymphoma,[4–6] leukemia,[7] melanoma,[8] and lung adenocarcinoma[9] cells. Acute toxicity tests using normal rats indicated that hybrid liposomes were free from any toxic action.[10] Furthermore, hybrid liposomes including antitumor drugs have been found to have a highly inhibitory effect on the growth of glioma in vitro and in vivo.[11] Significantly prolonged survival was obtained using mice model of carcinoma in vivo.[8]

It is well known that hydrophilic peptides like insulin are not sufficiently encapsulated to the liposomes. In order to improve the encapsulation of insulin into liposomes, we prepared the acylated insulins modified with hydrophobic groups, that is, long chain caproyl (Cap) and aromatic benzoyl (Bz) groups. In this study, we report the stability of the acylated insulins (Cap-insulin and Bz-insulin) in various digestive enzyme solutions and the hypoglycemia effects in the animal experiments using normal rats.

Insulin consists of two peptide chains, A chain is composed of 21 amino acid residues and B chain is 30 residues. It is possible to introduce acyl groups into the α-amino groups of N-terminal residues in each chain, Gly-A1 and Phe-B1, and ε-amino group of 29th residue in B chain, Lys-B29. Lindsay and Shall reported that the introduction of acetoacetyl or thiazalidinecarbonyl group into the Gly-A1 residue stimulated the decrease in the biological activity.[12] So, we prepared the acylated insulins by the reaction of insulin protected by *p*-methoxybenzoxy car-

Keywords: Acylated insulin; Diabetes; Digestive enzyme; Hypoglycemia.
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doi:10.1016/j.bmcl.2003.10.037

bonyl azide at Gly-A1 site with *N*-hydroxysuccinimide ester of caproic acid or benzoic acid as described previously.[13] The products were a mixture of mono and diacylated insulins at Phe-B1 and/or Lys-B29 residues, Bz-insulin and Cap-insulin (Fig. 1), which were confirmed on HPLC.[14]

Firstly, we examined the stability of acylated insulins to the various digestive enzymes (pepsin, chymotrypsin and carboxypeptidase) in vitro. Figure 2 shows the remaining insulins in the enzyme solutions at 37 °C after 4 h.[15] With respect to the acylated insulins, no degradation of Cap-insulin and Bz-insulin was observed in all enzyme solutions, though the native insulin was decomposed over 50%. This result suggests that the introduction of Cap group or Bz group into insulin could suppress the decomposition by proteolytic enzymes, and as a result the stability to the enzymatic hydrolyses in the digestive tract was improved.

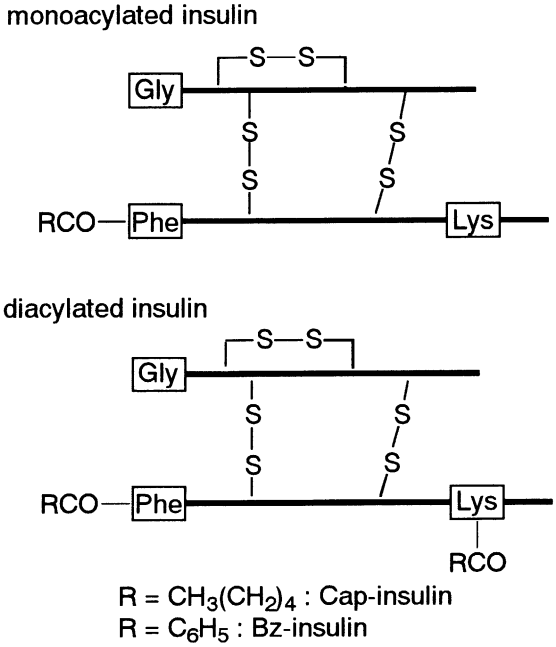

Figure 1. Structures of mono and diacylated insulins modified with caproyl or benzoyl group (Cap-insulin and Bz-insulin).

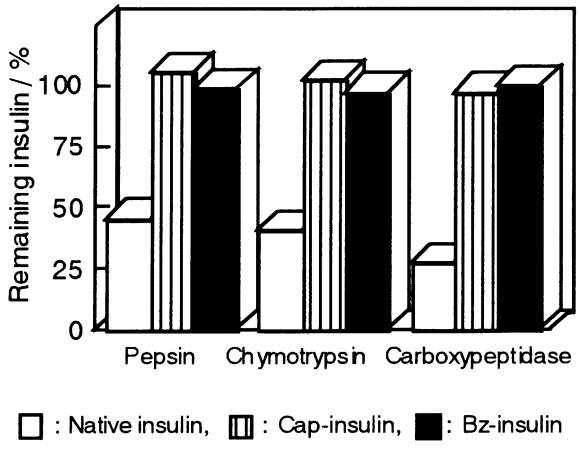

Figure 2. Decomposition of native and acylated insulins in various digestive enzyme solutions. [insulin] = 20 U mL^{-1}, [pepsin] = 5.0×10^{-3} g mL^{-1}, [chymotrypsin] = 1.0×10^{-3} g mL^{-1}, [carboxypeptidase] = 1.0×10^{-2} g mL^{-1}.

Secondly, we investigated the hypoglycemia effect of the acylated insulins using normal rats in vivo. The time course of change in glucose concentration in blood plasma of normal rats injected intravenously with the insulin solutions was monitored,[16] as shown in Figure 3. The biological activity of acylated insulins was calculated by the Eq. 1 using the area under the glucose concentration–time curve for 4 h after the administration ($AUC^{0-4\ h}$).[13]

$$\text{biological activity (\%)} = (AUC^{0-4\,h} \text{ of acylated insulin} / AUC^{0-4\,h} \text{ of native insulin}) \times 100 \quad (1)$$

In comparison with the native insulin ($AUC^{0-4\ h} = 206$), the biological activity of Cap-insulin was decreased to about 56% ($AUC^{0-4\ h} = 116$), which is still efficacious. On the other hand, $AUC^{0-4\ h}$ of Bz-insulin was almost similar to that of native insulin ($AUC^{0-4\ h} = 205$). That is, Bz-insulin is effective for retaining the hormonal activity. This result suggests that the Bz-group in the acylated insulin would not affect the affinity to the receptor.

In conclusion, it was clarified for the first time that the acylation of insulin with caproyl or benzoyl group improved the stability to the decomposition by various digestive enzymes. It was noteworthy that the hypoglycemia effects of Bz-insulin almost similar to that of native insulin was obtained in the animal experiments using normal rats in vivo. This result suggests that the acylated insulins should be a promising effective medicine of oral administration in clinical application of IDDM.

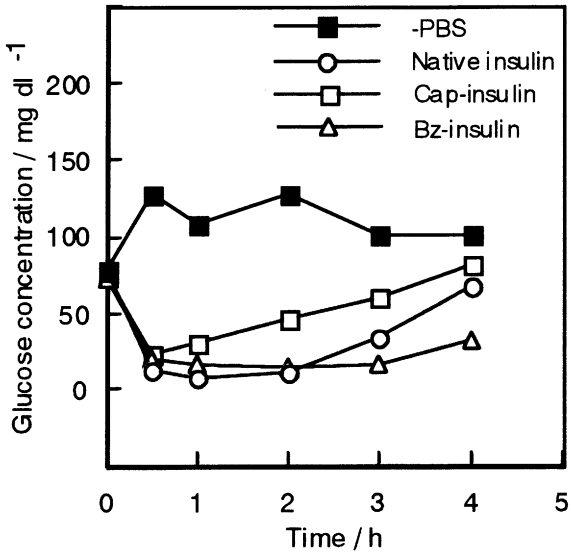

Figure 3. Time change of glucose concentration in blood plasma of normal rats after the injection intravenously with the insulin solutions.

Acknowledgements

We are grateful for Professor Eiichi Araki of Kumamoto University School of Medicine for helpful discussion.

References and notes

1. Kahn, C. R.; Weir, G. C. *Joslin's Diabetes Mellitus,* 13th ed.; Lea & Febiger, 1994; 194.
2. Yamamoto, A.; Muranishi, S. *Drug Delivery System* **1997**, *12*, 381.
3. Ueoka, R.; Matsumoto, Y.; Moss, R. A.; Swarup, S.; Sugii, A.; Harada, K.; Kikuchi, J.; Murakami, Y. *J. Am. Chem. Soc*. **1988**, *110*, 1588.
4. Ueoka, R.; Matsumoto, Y.; Oyama, H.; Takekuma, H.; Iio, M. *Chem. Pharm. Bull*. **1988**, *36*, 4640.
5. Ueoka, R.; Matsumoto, Y. *Bio Industry* **1993**, *10*, 221.
6. Matsumoto, Y.; Imamura, C.; Iio, T.; Taniguchi, C.; Ueoka, R. *Biol. Pharm. Bull*. **1995**, *18*, 1456.
7. Matsumoto, Y.; Kato, Y.; Kemura, Y.; Tsuchiya, M.; Yamamoto, M.; Ueoka, R. *Chem. Lett*. **1999**, 53.
8. Kanno, A.; Kodama, R.; Terada, Y.; Matsumoto, Y.; Ueoka, R. *Drug Delivery System* **1998**, *13*, 101.
9. Matsumoto, Y.; Kato, T.; Iseki, S.; Suzuki, H.; Nakano, K.; Iwahara, M.; Ueoka, R. *Bioorg. Med. Chem. Lett*. **1999**, *9*, 1937.
10. Imamura, C.; Kanno, A.; Mitsuoka, C.; Kitajima, S.; Inoue, H.; Iwahara, M.; Matsumoto, Y.; Ueoka, R. *Yakugaku Zasshi* **1996**, *116*, 942.
11. Kitamura, I.; Kochi, M.; Matsumoto, Y.; Ueoka, R.; Kuratsu, J.; Ushio, Y. *Cancer Res*. **1996**, *56*, 3986.
12. Lindsay, D. G.; Shall, S. *Biochem. J*. **1971**, *121*, 737.
13. Hashimoto, M.; Takada, K.; Kiso, Y.; Muranishi, S. *Pharm. Res*. **1989**, *6*, 171.
14. Nakazawa, H.; Nagase, M. *Yakugaku Zasshi* **1986**, *106*, 398.
15. Decomposition of native and acylated insulins by hydrolytic enzymes were examined in the aqueous solutions at pH 1.4 (pepsin) and pH 6.8 (chymotrypsin and carboxypeptidase). The remaining insulin in the solutions was monitored by measuring on a column of HPLC as described previously.[14]
16. Native and acylated insulins were dissolved in phosphate-buffered saline (-PBS) solution (10 U mL^{-1}) and filtered in a sterile manner through a 0.45 μm filter. The insulin solutions were injected to normal male Wistar rats (10 U kg^{-1}) through the tail vein after fasting for 18 h. The rats were anesthetized with ether and the blood was collected periodically from the orbit vein. The sample bloods were added to heparin (50 IU mL^{-1}) and centrifuged (2500 rpm) for 25 min at room temperature. The glucose concentration in blood plasma was measured by glucose oxidase method using Fuji DRI-CHEM 3500V (FUJIFILM MEDICAL).