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Synthesis and evaluation of photolabile insulin prodrugs

Lian-Sheng Li,[a,†] Jennie L. Babendure,[b,†] Subhash C. Sinha,[a,*] Jerrold M. Olefsky[b,*] and Richard A. Lerner[a,*]

[a]*The Skaggs Institute for Chemical Biology and Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[b]*Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92037, USA*

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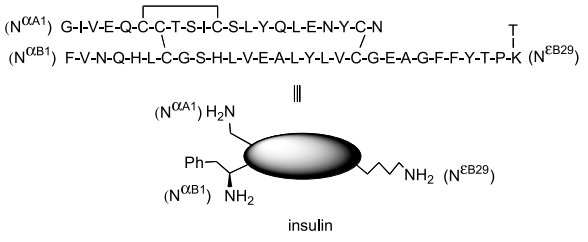
Abstract—We have developed two photolabile insulin prodrugs, insulin-2P and insulin-3P. These prodrugs were synthesized by protecting GlyA1 ($N^{\alpha A1}$), and one or both of the PheB1 ($N^{\alpha B1}$) and LysB29 ($N^{\varepsilon B29}$) amino groups in insulin using 5′-(α-methyl-nitropiperonyl)oxy-carbonyl as the protecting group. These insulin prodrugs were efficiently activated by exposure to longwave UV light to produce insulin quantitatively. Using 2-deoxyglucose uptake assays, both di- and tri-protected compounds were less active than native insulin in the protected state, and showed comparable activity to native insulin upon photoactivation.

1. Introduction

Hyperglycemia is the etiologic cause of most of the complications of diabetes mellitus. To prevent or reduce these complications, people with diabetes must maintain glycemic control as close to normal as possible. This often requires multiple daily injections of insulin as well as frequent self-glucose monitoring to maintain euglycemia. Patients often have great difficulty adhering to this rigorous schedule, and therefore it has long been a goal in diabetic treatment to establish a closed-loop system of regulated insulin delivery in response to elevated blood glucose levels. In hopes of making steps toward this goal, we have developed two insulin prodrug compounds by protecting the primary amines of human insulin with photolabile groups. These compounds are minimally active in the protected state, but become active after photolysis with 365 nm light. These compounds have the potential for use in an implantable closed-loop device, coupling a glucose sensor to a small UV lamp to photoactivate and release the correct dose of insulin in response to elevated glucose levels. The advantage of using photolabile compounds in a closed-loop system is that they will facilitate the development of smaller, simpler, and less invasive devices than insulin pump-based methods could provide. As the photolabile insulin is released by light rather than a pump, there would be no moving parts to replace, and accidental release of the prodrug would be less damaging to the body than accidental release of fully active insulin. In the present study, we describe the synthesis of these insulin prodrugs and evaluate their ability to stimulate glucose uptake in an in vitro system.

The insulin molecule consists of two polypeptide chains, A and B, joined together by two disulfide bonds. In addition to two N-terminal primary amines, A1 and B1, the insulin molecule also contains one ε amine on the lysine residue of chain B. To date, most insulin prodrugs or derivatives have been synthesized by protecting the primary amines at A1, B1, or B29.[1]



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* Corresponding authors. Tel.: +1 858 7848512; fax: +1 858 7848735 (R.A.L); Tel.: +1 858 5346651; fax: +1 858 5346653 (S.C.S.); Tel.: +1 858 7848265; fax: +1 858 7849899 (J.M.O.); e-mail addresses: subhash@scripps.edu; jolesfsky@ucsd.edu
† These authors contributed equally to this work.

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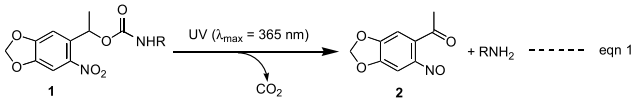
Studies have revealed that the PheB1 ($N^{\alpha B1}$) and LysB29 ($N^{\varepsilon B29}$) amino groups of insulin do not directly participate in receptor binding.[2] Thus, protection of these two amino groups has minimal effect on insulin activity. The GlyA1 ($N^{\alpha A1}$) amino group of insulin, however, plays a critical role in receptor binding and its protection leads to diminished binding affinity and biological activity. Utilizing this property of insulin, various derivatives have been synthesized. We previously investigated the functional properties of native insulin in comparison to an aldol-derivatized organoinsulin prohormone in the absence and presence of the catalytic aldolase antibody 38C2.[3] In the aldol-derivatized organoinsulin, all three amine functions of insulin were protected with aldol linkers. This prodrug compound exhibited markedly diminished receptor binding and biological activity both in vitro and in vivo. When the pro-insulin compound was incubated with 38C2, native insulin was regenerated, displaying restored receptor-binding affinity as well as normal in vitro and in vivo biological activity. Taking a cue from this study, we sought to design photolabile insulin prodrugs that could be activated by photolysis with near ultra violet (UV) light. Such photolabile insulin prodrugs could be stored in a receiver and photoactivated for administration. As a proof of concept, we have synthesized two photolabile insulin derivatives and studied their properties, including activation with UV light and the effect of the photolabile insulins on glucose uptake, in vitro.

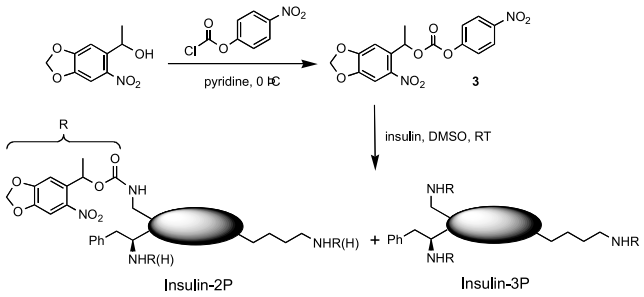
2. Synthesis, isolation, and activation of the photolabile insulins

For the synthesis of the photolabile insulins, we used the 5′-(α-methyl-nitro-piperonyl)oxy-carbonyl (MeNPOC) group to mask the free primary amines. It has been reported that the MeNPOC protecting group used in many applications, including the light-directed synthesis of DNA arrays on glass substrates, could be rapidly deprotected using UV light of 365 nm. As shown in Eq. 1, a compound with the general structure **1** is deprotected to the corresponding amine (RNH_2) and nitroso derivative **2** in less than a minute.[4] It is noteworthy that the 365 nm emission is almost exclusively responsible for the photochemistry, as the absorbance of the MeNPOC chromophore ($\lambda_{max} = 345$ nm, $\varepsilon = 5 \times 10^3\ M^{-1}\ cm^{-1}$) becomes negligible at wavelengths above 400 nm.



(1)

Synthesis of the N-protected insulins was achieved by treatment of parent insulin with carbonate **3** in DMSO followed by dialysis and Prep-HPLC purification (Scheme 1). Briefly, compound **3**[5] (3 equiv with respect to insulin) was added to a suspension of the recombinant human insulin (purchased from Sigma–Aldrich, 70 mg, 12 μmol) in reagent grade DMSO (3.0 ml). The resulting mixture was stirred in the dark at room temperature overnight. Then the crude mixture was ana-



Scheme 1. Protection of the primary amines in insulin using the photolabile groups to produce insulin prodrugs, insulins-2P and 3P.

lyzed by analytical HPLC assay using a reverse phase C18 column and a UV detector at 280 nm. Four major peaks at R_T 24.3, 27.7, 27.9, and 30.2 min were observed in the chromatogram (Fig. 1A). It was found that both insulin and 4-nitro-phenol generated from the reaction had the same retention time corresponding to 24.3 min (data not shown). Therefore the rest of the major peaks were expected to be modified insulins. The low molecular weight compounds (4-nitro-phenol, etc.) were removed completely after dialysis (Fig. 1B). Thus, the reaction mixture was diluted with H_2O (3.0 ml) and then transferred into a 3500 MW_{cutoff}, 12 ml dialysis membrane slide (Pierce) and dialyzed with either 0.1 N ammonium carbonate buffer or H_2O. The residue inside the membrane was chromatographed using preparative HPLC and two fractions were collected. The first fraction contained two inseparable compounds that corresponded to R_T 27.7 and 27.9 min, while the second fraction was a single compound corresponding to R_T

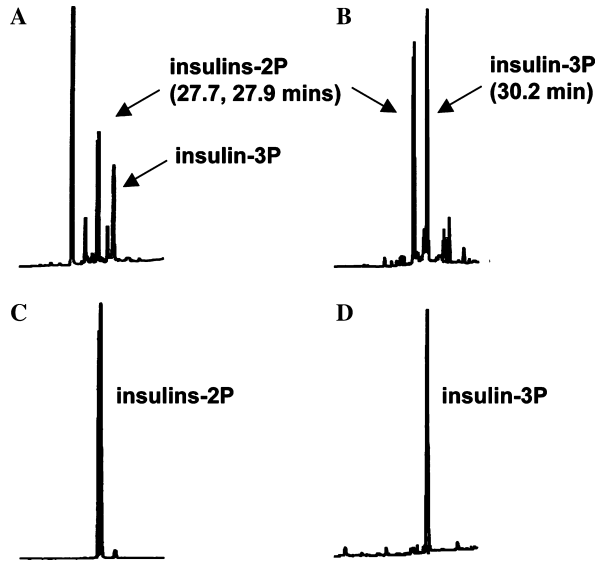
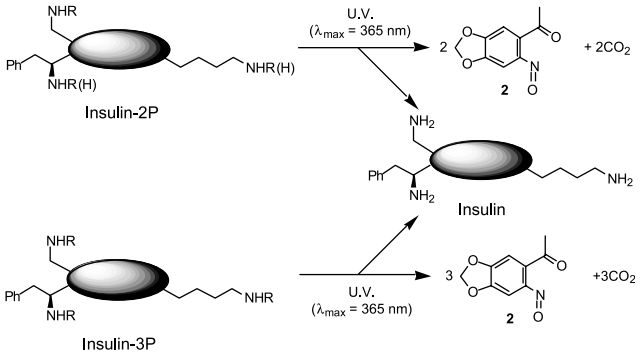


Figure 1. Sections of the HPLC traces showing: (A) the crude reaction mixture of insulin with compound **3**, (B) the reaction mixture shown in (A) after dialysis, and (C) the diprotected (insulin-2P) and (D) triprotected (insulin-3P) insulin derivatives separated by preparative HPLC. Conditions for the analytical HPLC: reverse phase C18 analytical HPLC column ($Vydac_{TM}$, Cat# 201SP54). Water–acetonitrile (100:0–20:80) at a rate of 0.8 ml/min using gradient system, starting with 100% water at 0 min and 80% acetonitrile in water at 40 min.

30.2 min in the analytical HPLC (Figs. 1C and D, respectively). Both fractions were separately lyophilized, to give 13 and 10 mg of the protected insulins.

The protected insulins were analyzed by liquid chromatography mass spectrometry (LCMS) electrospray ionization (ESI) experiments, which gave a molecular ion peak (M^+) at 6281 for the first fraction and at 6518 for the second fraction. As a control, recombinant human insulin showed a consistent molecular weight of 5807 under the same conditions. The data confirmed that the first fraction contained both diprotected insulins (insulins-2P), whereas the second fraction contained the triprotected insulin derivative (insulin-3P). Based on our previous study, it was evident that only the three primary amine functions at GlyA1 ($N^{\alpha A1}$), PheB1 ($N^{\alpha B1}$), and LysB29 ($N^{\varepsilon B29}$) could be protected in insulins-2P as well as 3P under the reaction conditions, however, it was not clear which two amines were protected in insulins-2P. Fortunately, the biological data suggest that the GlyA1 ($N^{\alpha A1}$) amino group, which directly participates in receptor binding, was protected in both diprotected compounds. Alternative protection of PheB1 ($N^{\alpha B1}$) and LysB29 ($N^{\varepsilon B29}$) amino groups made two diprotected insulins, which were inseparable by HPLC. The attempts to convert all the insulin to insulin-3P by increasing the amount of compound **3** and prolonging the reaction time were unsuccessful.

Next, we examined the activation of the insulin prodrugs using a high intensity longwave UV lamp (Ultra-Violet Products, model # B series 100AP). Both insulins-2P and 3P underwent rapid deprotection to release free insulin and the nitroso derivative **2** (Scheme 2) as evidenced by the HPLC (Fig. 2) and (LCMS) analyses of the crude products, using authentic insulin as comparison. As shown in Figures 2A and B, respectively, insulins-2P and 3P were converted to the free insulin (R_T 24 min) as well as the corresponding intermediates, which are most likely the mono-protected insulins ($R_T \sim 26$ min) from insulins-2P, and the mixtures of mono- and diprotected insulins ($R_T \sim 26$–28 min) from insulin-3P. Thus, at the end of the first minute of UV treatment, more than 50% and 30% insulin were generated from insulin-2P and 3P, respectively. The insulin concentration from insulins-2P and 3P increased to



Scheme 2. Activation of insulins-2P and 3P by photolysis under UV light (hν = 365 nm) to regenerate insulin.

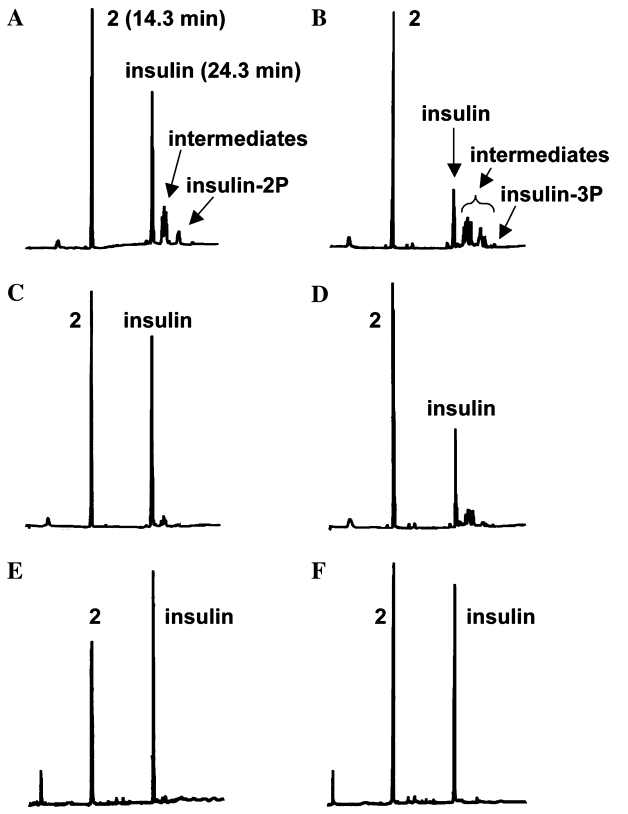


Figure 2. Part of the HPLC traces showing the regeneration of insulin and production of compound **2** from insulin-2P (A, C, and E) at the end of 1, 2, and 5 min and from insulin-3P (B, D, and F) at the end of 1, 2, and 10 min, respectively. (HPLC conditions: see Fig. 1).

75% and 50% at the end of second minute (Figs. 2C and D), and finally rose to almost complete deprotection at 5 and 10 min, respectively (Figs. 2E and F).

3. Prodrugs insulins-2P and 3P stimulate glucose uptake when photoactivated

To demonstrate the activity of insulin regenerated from insulins-2P and 3P, we performed 2-deoxyglucose uptake assays before and after photoactivation. Glucose uptake was determined by assessing the amount of [^{3}H]2-deoxyglucose taken up by cells stimulated with native insulin, protected, or photoactivated compounds. For this study, 3T3-L1 adipocytes were cultured and differentiated as previously described.[6] Cells were seeded into 12 well plates and the assay was conducted on days 12–14 of differentiation. Insulins-2P and 3P were protected from light and dissolved in 10 mM HCl before use. Dissolved compounds were placed in Acryl-cuvettes (Sarstedt), and illuminated for 15 min with a BLAK-RAY Longwave Ultraviolet Lamp (Model B-100A, San Gabriel, CA) to induce photolysis. Native human insulin, insulins-2P and 3P, were used at two doses, 0.5 and 17 nM. Glucose uptake was measured as described elsewhere,[6] with some adjustments. 3T3-L1 adipocytes were serum starved for 2 h and stimulated with insulin, the protected insulins-2P or -3P, or the photoactivated compounds for 20 min. Glucose uptake was

measured by incorporation of [^{3}H]2-deoxyglucose after a 15 min incubation.

Figure 3 compares glucose uptake using insulins-2P and 3P. Both compounds were moderately active in the protected state, perhaps indicating some degree of degradation. Upon photoactivation, the compounds demonstrated variable degrees of activity, with the diprotected insulin-2P activating 70% of the maximal response (Fig. 3A). The triprotected insulin-3P appeared to activate 116% of the maximal response; however, this difference from native insulin was not statistically significant as shown by one-way ANOVA followed by Dunnett's multiple comparison post-test (Fig. 3B). One explanation for the increased activity seen with both protected and deprotected insulin-3P is that a small portion of the compound may have been deprotected prior to measurement and use.

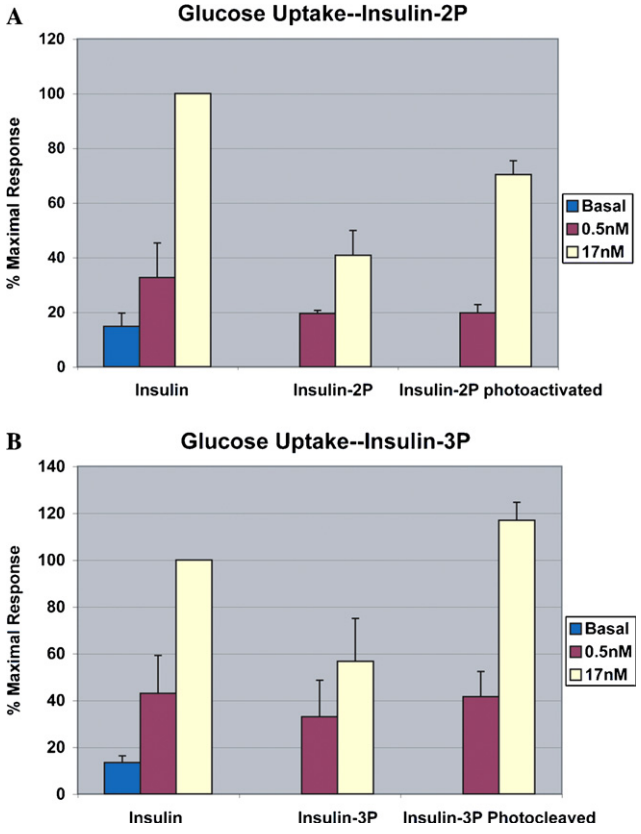


Figure 3. Glucose uptake stimulated by native insulin, protected and photoactivated insulin compounds. 3T3-L1 adipocytes were stimulated with the indicated amounts for 20 min and glucose uptake was quantitated by incorporation of [^{3}H]2-deoxyglucose. (A) Activity of insulin 2P in comparison to native insulin. (B) Activity of insulin 3P in comparison to native insulin. Figures are an average of three experiments.

In conclusion, photolabile insulin prodrugs, insulins-2P and insulin-3P, were developed by protecting GlyA1 ($N^{\alpha A1}$) and one or both of the PheB1 ($N^{\alpha B1}$) and LysB29 ($N^{\varepsilon B29}$) amino groups using MeNPOC as the protecting group. These insulin prodrugs were efficiently activated by exposure to longwave UV light to produce insulin quantitatively. Using 2-deoxyglucose uptake assays, both di- and tri-protected compounds were significantly less active than native insulin, and activity was restored upon photoactivation. These compounds provide a possible further step toward the development of a closed-loop system of concomitant glucose detection and insulin delivery.

Acknowledgments

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