E₂ (17β-oestradiol), a female sex hormone, has important biological functions in a woman’s body. The pancreas, often considered a non-classical E₂-targeting organ, is known to be functionally regulated by E₂, but little is known about how oestrogen actions are regulated in this organ. In the present study we report that PDIp (pancreas-specific protein disulfide isomerase), a protein-folding catalyst, can act as a major intracellular E₂ storage protein in a rat model to modulate the pancreatic tissue level, metabolism and action of E₂. The purified endogenous PDIp from both rat and human pancreatic tissues can bind E₂ with a Kᵦ value of approximately 150 nM. The endogenous PDIp-bound E₂ accounts for over 80% of the total protein-bound E₂ present in rat and human pancreatic tissues, and this binding protects E₂ from metabolic disposition and prolongs its duration of action.

Importantly, we showed in ovariectomized female rats that the E₂ level in the pancreas reaches its highest level (9-fold increase over its basal level) at 24–48 h after a single injection of E₂, and even at 96 h its level is still approximately 5-fold higher. In contrast, the E₂ level in the uterus quickly returns to its basal level at 48 h after reaching its maximal level (approximately 2-fold increase) at 24 h. Taken together, these results show for the first time that PDIp is a predominant intracellular oestrogen storage protein in the pancreas, which offers novel mechanistic insights into the accumulation and action of oestrogen inside pancreatic cells.

Key words: 17β-oestradiol (E₂), oestrogen, oestrogen receptor, pancreas, pancreas-specific protein disulfide isomerase (PDIp).

INTRODUCTION

E₂ (17β-oestradiol), an important endogenous female sex hormone, exerts biological functions in various target organs or tissues. The hormonal actions of E₂ are vital for the maintenance of normal female physiology, including the development of reproductive organs and secondary sex characteristics. Many of the well-known physiological actions of E₂ are mediated by the genomic actions of ER (oestrogen receptor) α and ERβ [1], which are master regulators of target gene transcription. In addition, the non-genomic actions of ERs have also been suggested to play a role in mediating E₂-induced rapid signal transduction in certain systems [2,3].

Besides the classical oestrogen target organs (e.g. breast, uterus, ovary and pituitary) [4], the pancreas, which is often considered a non-classical target organ, has been known for years to be functionally regulated by oestrogens. The pancreas has both endocrine glands, which produce hormones (e.g. insulin and glucagon), and exocrine glands, which secrete pancreatic juice containing digestive enzymes. It was reported previously that oestrogen can be biosynthetically formed in the canine pancreas [5], and that both ERα and ERβ are expressed in pancreatic islet cells and acinar cells [6–8], which produce and secrete insulin and digestive enzymes respectively. Providing further support for the functions of oestrogens in the pancreas, studies have shown that oestrogen can modulate insulin production and enhance islet cell survival and growth under certain conditions [9–14]. Similarly, oestrogen can also modulate the production of pancreatic digestive enzymes in pancreatic acinar cells [15–17]. In addition, studies have shown that pancreatic cancers have a higher prevalence in males than in females in both humans and animals [18–23], and it has been suggested that oestrogen may have a protective effect against pancreatic tumorigenesis [18–20,24].

The present study began with an unexpected observation showing that in ovariectomized female rats following administration of E₂, the pancreas can accumulate E₂ more persistently and abundantly than the uterus, a classical E₂ target organ. Following this observation, we also showed that the accumulation of E₂ in the pancreas is largely attributable to PDIp [pancreas-specific PDI (protein disulfide isomerase)], which has a high capacity for binding E₂ in vitro and in vivo and can also slow down E₂ metabolism in vitro. PDIp was previously reported to be a protein-folding catalyst [25–27], predominantly localized in the endoplasmic reticulum [28] and selectively expressed at high levels in pancreatic acinar cells [25,28–31]. The results of the present study indicate that besides serving as a protein-folding catalyst, PDIp may also have other biological functions through modulating pancreatic tissue levels and the metabolism of endogenous oestrogens. Accordingly, PDIp will be an important modulator of oestrogen biological actions in the pancreas.

EXPERIMENTAL

Chemicals, reagents, plasmids, cell lines and tissues

E₂ was purchased from Steraloids. [³H]E₂ (specific activity of 110 Ci/mmol) was obtained from PerkinElmer. Mastoparan...
(Ile-Asn-Leu-Lys-Ala-Leu-Ala-Leu-Ala-Leu-Ala-Lys-Lys-Ile-Leu) was obtained from Sigma–Aldrich. All other chemicals and reagents used in the present study were of analytical grade or higher. Specific rabbit antibodies against a number of proteins were used in the present study, and they were obtained from the following sources: anti-PDI was from Sigma–Aldrich (catalogue no. P7372, dilution of 1:2000 for Western blotting), anti-ERα (sc-543, 1:200 dilution) was from Santa Cruz Biotechnology and anti-ERβ (P17-7700, 1:100 dilution) was from Invitrogen. The mouse anti-PDIp antisera (dilution of 1:2500 for Western blotting) was raised in our laboratory [29]. The ERE (oestrogen response element)-driven luciferase vector (pGL3-Basic + ERE + E1b + luciferase) was a gift from Dr Carolyn Smith (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, U.S.A.). pcDNA3.1-PDIp was constructed as described previously [28]. The monkey kidney COS-7 cell line was purchased from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured in DMEM (Dulbecco’s modified Eagle’s medium) plus 10% (v/v) FBS (fetal bovine serum) (Gibco). The human pancreatic cancer cell line, BxPC-3, was purchased from Shanghai Institutes for Biological Science. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) FBS in a 37°C incubator with 5% CO2. The human pancreatic tissue specimen was obtained from the National Disease Research Interchange (catalogue no. 0060960–13).

**Protein purification**

Natural PDIp from rat or human pancreas tissues were purified as described below. For each batch experiment, approximately 1.5 g of tissue was used. First, tissues were ground into fine powder in liquid nitrogen and then dissolved in 10 ml of 10 mM sodium phosphate buffer plus protease inhibitors (Sigma–Aldrich). After homogenization and centrifugation (10,000 g for 16 min at 4°C), the sample was separated into three layers, from top to bottom: floating lipids, soluble fraction and insoluble pellets. After removing the lipid layer carefully, the soluble fraction was filtered with a 0.8 µm-pore-size Millex-CA Filter Unit (Millipore) and then loaded on to a Superdex 200 10/300 GL column (GE Healthcare) for SEC (size-exclusion chromatography), which was performed on an AKTA FPLC system and eluted with 10 mM sodium phosphate buffer (pH 7.4) at a flow-rate of 0.6 ml/min at room temperature (25°C). The fractions were collected at 1 ml/tube in an ice bath. SEC was repeated 4–5 times in each batch experiment. The fractions containing PDIp were combined and further separated by IEC (ion-exchange chromatography) using a Mono Q 4.6/100 PE column (GE Healthcare). During IEC, the column, elution buffer and collecting tubes were kept in an ice bath. The typical salt-gradient elution was performed from 0 to 0.1 M NaCl (10 mM sodium phosphate, pH 7.4) in 1 column vol. and then gradually to 0.3 M NaCl in 12 column vol. followed by increasing to 1.0 M NaCl in 2 column vol.

**[3H]E2-binding assay for purified PDIp proteins and live mammalian cells**

We explored the desalting procedure to separate free [3H]E2 and protein-bound [3H]E2 as described previously [32]. Non-specific [3H]E2-binding was determined in the presence of 10 µM E2. The [3H]E2-binding of COS-7 cells (seeded in a 48-well plate) was measured as described in our previous study [29]. The non-specific [3H]E2-binding was determined in the presence of 20 µM E2.

**Animal experiments**

Female ovariectomized Sprague–Dawley rats (7 weeks old) (from Harlan Sprague–Dawley Laboratory, Houston, TX, U.S.A.) were used in the present study. The animal use procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center, and the NIH guidelines for humane treatment of animals were carefully followed. After arrival, they were allowed to acclimatize for 1 week before the experiments. Each rat was administered (intraperitoneal injection) with 500 µl of corn oil containing 0.13 mg E2. At each time point (24, 48, 72 and 96 h), three rats were subjected to blood perfusion with saline buffer (0.7% NaCl) after killing with isoflurane. Tissues (pancreas, liver and uterus) were collected and immediately stored at −80°C until used for E2 extraction, and 4 ml of serum was also collected for E2 extraction.

**Transfection of cells and oestrogen signalling assay**

BxPC-3 cells were cultured (usually overnight) with RPMI 1640 medium plus 10% (v/v) FBS in 24-well plates to reach approximately 50% confluence before transfection. The plasmid pGL-Basic + ERE + E1b + luciferase and pcDNA3.1-PDIp (pcDNA3.1 used as a control) were transfected together using Lipofectamine™ 2000 and Opti-MEM I reduced serum medium (both from Invitrogen). At 24 h after transfection, cells were cultured with 600 nM E2 for 2 h, washed once and then cultured with the RPMI 1640 medium plus 10% dextran charcoal-treated FBS for another 4 days. Cell lysates at the indicated time points were subjected to luciferase activity assay (using the E1500 kit from Promega) and protein concentration determination.

**E2 extraction and concentration assay**

Rat tissues were weighed, ground into fine powder in liquid nitrogen and then homogenized in water. Lysates were then mixed thoroughly with 4 vol. of methylene chloride and centrifuged at 7000 g for 5 min. The methylene chloride layers were transferred into glass tubes, dried with nitrogen gas and then extracted with 20% ethanol to remove excessive lipids. After centrifugation at 3500 rev./min for 10 min, the clean layer was collected, dried and dissolved in water in glass tubes. After sonication in the water bath of a Fisher Scientific sonicator for 15 min, samples were subjected to an E2 concentration assay using an ELA (enzyme immunoassay) kit (Cayman Chemical Company) according to the manufacturer’s instructions.

**In-vitro metabolism of E2**

The metabolism of E2 by human liver microsomes was performed as described previously [33]. Briefly, E2 (3.6 nM [3H]E2 plus 2500 nM non-radioactive E2, final concentration) was incubated with pancreas tissue (5 mg/ml) at room temperature for 2 h in 250 µl of reaction buffer (0.1 M Tris/HCl, pH 7.4, and 0.05 M Hepes) in the absence or presence of 50 µM mastoparan in a glass tube and then kept on ice for 10 min. A 100 µl portion of a mixed solution containing human liver microsomes (0.36 mg/ml), 2 mM NADPH and 5 mM ascorbic acid was added quickly into each tube (final volume 350 µl) and gently mixed on ice. The reaction was performed in a 37°C water-incubator with gentle shaking. At 8 min later, the reaction was stopped by quickly placing the tubes in an ice bath and then immediately extracted with 5 ml of ethyl acetate. The oestrogen metabolites were dissolved in methanol and subjected to HPLC analysis using the same
Modulation of pancreatic E₂ level by PDIp

Figure 1 PDIp accumulates E₂ in the pancreas of rats

(A) Basal concentrations of E₂ in the pancreas and uterus of untreated ovariectomized female rats. E₂ was extracted from tissues and then subjected to RIA analysis of its concentration as described in the Experimental section. (B) E₂ concentrations in the pancreas and uterus of ovariectomized female rats that received a single i.p. injection of E₂ (0.13 mg/rat, dissolved in corn oil) and then sacrificed at the indicated time points (24, 48, 72 and 96 h). The control rats were injected with corn oil only. Results are means ± S.D. (n = 5).

conditions as described previously [33]. The metabolism ratio was calculated by dividing the radioactivity of all metabolites with the total radioactivity of substrate.

RESULTS

Accumulation of E₂ in the pancreas: evidence from in vivo animal studies

Using ovariectomized female rats as an in vivo model, in which the endogenous oestrogen biosynthesis is mostly absent, we found that the basal levels of E₂ in the pancreas were 30 ± 10 pg per g of tissue, which are approximately one-fifth of the basal levels (125 ± 40 pg per g of tissue) found in the uterus, a classical oestrogen target organ (Figure 1A). This difference presumably is due to the higher levels of ERα and ERβ present in the uterus, which can help accumulate more endogenous oestrogens when their concentrations are extremely low.

After a single intraperitoneal administration of E₂ to these ovariectomized female rats, the uterine growth (reflected by uterine wet mass gain) was stimulated in a time-dependent manner, indicating a strong hormonal effect of the administered E₂ in the uterus (Supplementary Figure S1 at http://www.BiochemJ.org/bj/447/bj4470115add.htm). When the uteri from these animals were removed for measuring the tissue levels of E₂, we found that E₂ in the uterus was increased by approximately 100 % at 24 h after E₂ administration, but at 48 h, it returned to its original basal level (Figure 1B). In contrast, the changes in pancreatic E₂ level were very different. The pancreatic E₂ level increased dramatically (approximately 900 %) at the 24 h after E₂ administration and, following its rapid initial rise, its levels decreased very slowly compared with the uterus. Surprisingly, the E₂ level remained elevated, at approximately 500 % higher.

Figure 2 Natural PDIp in rat pancreas has E₂-binding activity

(A) The measurement of protein level (left-hand y-axis) and the bound [³H]E₂ (right-hand y-axis) for each chromatographic fraction of the rat pancreatic tissue lysates separated by the Superdex-200 gel filtration column (see Supplementary Figure S2A at http://www.BiochemJ.org/bj/447/bj4470115add.htm). (B) Western blot analysis of ERα, ERβ, PDI and PDIp in each collected elution fraction from (A). Note that only 2 μl of the sample from each fraction was loaded on to the SDS gel for detection of PDIp and PDI, whereas 20 μl of the sample was loaded for detection of ERα and ERβ. (C) Protein level (left-hand y-axis) and bound [³H]E₂ (right-hand y-axis) of each fraction collected from the Mono-Q column IEC (see Supplementary Figure S2B at http://www.BiochemJ.org/bj/447/bj4470115add.htm), by which fractions A7 – A8 from (A) were separated. (D) Western blot analysis of ERα, ERβ, PDI and PDIp in fractions B2, B3 and B4 from (C). Note that although the sample amount used for Western blot analysis of ERα, ERβ and PDI was the same as that for Coomassie Blue staining, the amount for Western blot analysis of PDIp was only one-twentieth of that used for Coomassie Blue staining. (E) SDS/PAGE analysis (visualized by Coomassie Blue staining) of fractions B2, B3 and B4 from (C). The band for PDIp is indicated.

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than its initial basal level, even at 96 h after a single \( E_2 \) injection (Figure 1B).

Identification of PDIp as the most abundant \( E_2 \)-binding protein in rat pancreas

The above in vivo animal study suggests that the pancreas has the unique ability to accumulate large amounts of \( E_2 \). We hypothesized that there might be one or more \( E_2 \)-binding protein(s) present in the rat pancreas, which is (are) distinct from the \( \text{ER} \alpha \) and \( \text{ER} \beta \), but can store large amounts of \( E_2 \). To identify such \( E_2 \)-binding protein(s), we employed a two-step chromatography approach to isolate proteins, which were then subjected to an in vitro radiometric [\(^{3}H\)]\( E_2 \)-binding assay. The rat pancreatic tissue lysates were first subjected to SEC (Supplementary Figure S2A at http://www.BiochemJ.org/bj/447/bj4470115add.htm). Next, the [\(^{3}H\)]\( E_2 \)-binding assay showed that fraction A7 had much higher \( E_2 \)-binding activity than other fractions, whereas fraction A8 also had some \( E_2 \)-binding activity (Figure 2A). Western blotting analysis of these fractions showed that \( \text{ER} \alpha \) and \( \text{ER} \beta \) are most abundantly present in fractions A4, A5 and A6, but not in A7 and A8 (Figure 2B), suggesting that non-\( \text{ER} \alpha /\text{ER} \beta \) proteins in fractions A7 and A8 account for their high \( E_2 \)-binding activity.

Next, we hypothesized that PDI and its pancreas-specific homologue (PDIp) might account for the high \( E_2 \)-binding activity in the rat pancreas on the basis of the following reasons. First, previous studies by others [24,29–31] and also by us [26,32,33] have shown that PDI and PDIp have \( E_2 \)-binding activity in vitro. Secondly, although PDI is abundantly expressed in many tissues [28,34], PDIp is highly and selectively expressed in the pancreas, at an estimated concentration of up to 0.5% of the total cellular proteins [25,28]. Thirdly, the molecular sizes of PDI and PDIp appear to be comparable with the sizes seen at the SEC elution volumes for fractions A7 and A8 (Figure 2A and Supplementary Figure S2). Fourthly, both PDI and PDIp are intracellular soluble proteins, in contrast with the extracellular proteins (such as steroid hormone-binding globulins) [35] and the membrane-associated \( E_2 \)-binding proteins.

To test this hypothesis, a number of experiments were performed. Western blotting analysis of PDI and PDIp proteins showed that they are predominantly present in fraction A7 (lane 6 in Figure 2B) and to a lesser extent also in fraction A8 (lane 7) and A9 (for PDI). To determine whether PDI or PDIp accounts for the \( E_2 \)-binding activity in SEC fractions A7 and A8, IEC was used to further separate PDIp from PDI in fractions A7 and A8 (Supplementary Figure S2B). A [\(^{3}H\)]\( E_2 \)-binding assay indicated that fraction B3 has the highest [\(^{3}H\)]\( E_2 \)-binding activity (Figure 2C). Western blotting analysis showed that PDIp is predominantly present in fractions B2, B3 and B4, whereas \( \text{ER} \alpha \), \( \text{ER} \beta \) or PDI proteins were not detected (Figure 2D, lanes 1–3). Notably, when the SDS gel was stained with Coomassie Blue, only one dominant band in these fractions was observed, with its molecular size matching closely that of PDIp and its position matching the band in the Western blotting gel (Figure 2E). Collectively, these results indicate that PDIp, but not PDI, \( \text{ER} \alpha \) or \( \text{ER} \beta \), accounts mostly for the [\(^{3}H\)]\( E_2 \)-binding activity of the IEC fractions.

Figure 3  Natural PDIp in human pancreatic tissue has \( E_2 \)-binding activity

The experimental strategy and procedures for the results shown in this Figure were essentially the same as those shown in Figure 2.
Identification of PDIp as the most abundant E2-binding protein in human pancreas

Using a similar strategy, we also purified PDIp from human pancreas by jointly using SEC (Supplementary Figure S2A) and IEC (Supplementary Figure S2C). A [3H]E2-binding assay showed that SEC fraction A7 has the highest [3H]E2-binding activity among all of the SEC fractions collected (Figure 3A), and the IEC fraction B3 has the highest [3H]E2-binding activity among all of the IEC fractions collected (Figure 3C). Western blot analysis showed that PDIp is predominantly present in SEC fraction A7 and to a lesser extent in fraction A8 (Figure 3B), and that PDIp is predominantly present in IEC fraction B3 whereas ERα, ERβ and PDI proteins are not detected in the IEC fraction B3 (Figure 3D). Furthermore, when the SDS gel was stained with Coomassie Blue, only one band with a molecular size matching that of PDIp was observed in the IEC fraction B3, and this band was marginally detected in fractions B2 and B4 (Figure 3D). In another IEC experiment, in which the salt elution condition was modified (Supplementary Figure S3A at http://www.BiochemJ.org/bj/447/bj4470115add.htm), only PDIp was detected in those fractions with [3H]E2-binding activity, whereas PDI, ERα and ERβ were not detected in these fractions (Supplementary Figures S3B and S3C). Collectively, these observations show that PDIp, but not PDI, ERα or ERβ, accounts for the [3H]E2-binding activity in human pancreas tissue.

PDIp proteins isolated from rat and human pancreas have similar E2-binding affinity

Next, we further sought to determine the E2-binding affinity of rat and human PDIp proteins that were highly purified as described above. A radiometric binding assay showed that the [3H]E2-binding affinity was 188 ± 43 nM (Figure 4A) and 147 ± 18 nM (Figure 4B) respectively. In comparison, under the same experimental conditions, the Kd values of the purified ERα and ERβ for [3H]E2 were 2.6 ± 0.5 nM and 3.8 ± 1.2 nM respectively (Figure 4C). The striking differences in E2-binding affinity between the IEC fraction B3 and the purified ERα and ERβ proteins also indicate that the [3H]E2-binding activity of the IEC fraction B3 is not attributable to the potential contamination of ERα or ERβ protein. Notably, the E2-binding affinity for the purified recombinant human PDIp (expressed in mammalian cells) determined in our recent study [32] was 170 ± 14 nM, which is very close to the E2-binding affinity of the purified endogenous rat and human PDIp protein as determined in the present study. This result further supports the conclusion that the E2-binding activity of the IEC fraction B3 is mostly attributable to PDIp rather than to other low-abundance proteins contained in this fraction.

In vitro accumulation of E2 by PDIp in pancreatic tissue lysates

The results from the protein isolation and radiometric E2 binding experiments described above (Figures 2 and 3) showed that PDIp accounts for most of the total [3H]E2-binding activity in pancreatic tissue lysates. Next, we performed additional experiments to further verify this finding. First, we measured the PDIp-specific [3H]E2-binding activity in the total tissue lysates prepared from rat, monkey or human pancreas. The effect of endogenous ERα and ERβ was masked by the presence of a saturating concentration of tamoxifen, which can compete with [3H]E2 for binding to ERα and ERβ (Figure 5B) [36,37], but not for binding to PDIp (Figure 5B) [32]. Taking advantage of this unique property of tamoxifen, we observed that although a majority of the total [3H]E2-binding activity of human, monkey or rat pancreatic tissue lysates prepared [3H]E2-binding activity of human, monkey or rat pancreatic tissue lysates could be competed off by the presence of excess E2, the presence of tamoxifen only very modestly reduced the total binding (Figure 5A). Accordingly, the quantitative difference between E2 competition and tamoxifen competition would give PDIp-specific [3H]E2-binding, which is over 80% of the total [3H]E2-binding activity of the pancreatic tissue lysates prepared
from these three different species. In contrast, the $[^3H]E_2$-binding activity of the tissue lysates prepared from rat liver and uterus was suppressed almost equally by the presence of excess $E_2$ or tamoxifen, clearly suggesting that their $[^3H]E_2$-binding activity is mostly from the oestrogen receptors (ER$\alpha$ and ER$\beta$).

To further probe this question, we also examined the effect of mastoparan, a small peptide that has opposite binding properties to tamoxifen, i.e. it can selectively bind to PDlp, but cannot bind to the oestrogen receptors (ER$\alpha$ and ER$\beta$). As expected, mastoparan was found to effectively compete with $[^3H]E_2$ for binding to PDlp (Figure 5B; or refer to our recent study [32]), but not its binding to ER and ER$\beta$ (Figure 5B). Taking advantage of this unique property of mastoparan, we observed that the total $[^3H]E_2$-binding activity of human, monkey and rat pancreatic tissue lysates was mostly abolished when excess mastoparan was present (Figure 5A). In contrast, the total $[^3H]E_2$-binding activity of liver and uterus tissue lysates was not appreciably affected by the presence of excess mastoparan (Figure 5A). These observations collectively demonstrate that the total $E_2$-binding activity of pancreatic tissues is mostly (over 80%) attributable to PDlp, but not ER$\alpha$ and/or ER$\beta$.

**Accumulation of $E_2$ by PDlp in live COS-7 cells**

In the present study, we also expanded our previous observation that PDlp is able to accumulate $E_2$ in live COS-7 cells [29], by further examining this ability using a wider range of $E_2$ concentrations and also comparing the specific- and non-specific bindings of $E_2$. Live COS-7 cells that over-expressed PDlp were found to accumulate $[^3H]E_2$ present at various concentrations (from 25 to 1000 nM) (Figure 5D). The magnitude of accumulation (i.e. the ratio of specific binding to non-specific binding) was as high as 6-fold when $[^3H]E_2$ concentrations were below 200 nM (Figure 5E), suggesting that the over-expressed PDlp has a stronger $E_2$-accumulating effect when the extracellular $E_2$ levels are relatively lower. In contrast, COS-7 cells transfected
with an empty vector could not accumulate \(^{3}H\)E\(_2\), as reflected by the lack of appreciable differences between the total and nonspecific \(^{3}H\)E\(_2\)-binding activity (Figure 5C).

**Modulation of PDIp on E\(_2\) in vitro**

One of the biological consequences of the PDIp–E\(_2\) binding interaction is that PDIp-bound E\(_2\) would not be accessible by the intracellular oestrogen-metabolizing enzymes [5] and, as such, the oestrogens are partially protected from metabolic disposition. Experimentally, we performed the in vitro metabolism of E\(_2\) catalysed by human liver microsomes and examined the effect of PDIp that is present in pancreatic tissue lysates. Again, taking advantage of the fact that mastoparan can compete with E\(_2\) for binding to PDIp (Figure 5B), we showed that the metabolic rate of E\(_2\) was increased significantly due to the presence of mastoparan (Figure 6A), whereas the presence of mastoparan alone did not significantly affect the metabolism of E\(_2\) (results not shown). These observations indicate that the binding of E\(_2\) by PDIp present in pancreas tissue lysates can delay its metabolism in vitro.

Next, we examined the modulating effect of PDIp on the actions of E\(_2\). Our previous study showed that human pancreatic cancer cells (e.g. BxPC-3, Mia PaCa-2 and Capan-2) do not express endogenous PDIp [28], but they express oestrogen receptors [38]. Hence we chose to use the approach of selectively overexpressing PDIp in BxPC-3 cells, coupled with concomitant transfection of an ERE-driven luciferase reporter gene. These cells were then used to examine the modulating effect of PDIp on oestrogen action. We observed that selective overexpression of PDIp (Figure 6B, inset) dramatically prolonged the duration of E\(_2\) action in these cells (Figure 6B), presumably due to increased intracellular accumulation of E\(_2\) plus a reduction in its metabolism.

In light of these observations, in the present study we also determined whether there is a sex difference in PDIp expression level in the pancreas of rats. However, no appreciable difference in PDIp protein level was observed in female and male rats (Figure 6C, lanes 1–6). Because the endogenous oestrogen levels in males are usually far lower than those in females, it is possible that the PDIp-mediated accumulation of intracellular E\(_2\) and its modulation of oestrogen metabolism and action are probably more important in females than in males.

**DISCUSSION**

A number of novel findings have been made in the present study, which jointly support the hypothesis that PDIp is a quantitatively major intracellular protein that can help accumulate large amounts of E\(_2\) in the pancreas. First, purified PDIp proteins from rat and human pancreas tissues have E\(_2\)-binding affinity in vitro (Figure 4). Secondly, PDIp accounts for most of the E\(_2\)-binding activity during chromatographic isolation of pancreatic tissue lysates (Figures 2 and 3), and the PDIp-bound E\(_2\) in pancreas tissue lysates accounts for approximately 80% of the total protein-bound oestrogen (Figure 5A). Thirdly, PDIp overexpressed in mammalian cells can accumulate E\(_2\) present at a wide concentration range (Figure 5D). Fourthly, our previous study reported that the protein level of PDIp in animal pancreatic tissues is as high as 0.5% of the total cellular proteins [29]. Jointly, these properties of PDIp would enable it to serve as a predominant oestrogen-binding and storage protein in the pancreas. This notion is further supported by observations from the animal experiments (Figure 1B) showing that the concentration of E\(_2\) in the pancreas of ovariectomized female rats is elevated following a single injection of E\(_2\), and this increase in pancreatic tissue levels of E\(_2\) is far higher and lasts far longer than the increase in E\(_2\) seen in the uterus.

On the basis of the findings of the present study, it is reasonable to suggest that PDIp may play a role in modulating the biological functions of endogenous oestrogen in the pancreas, partly through accumulating a higher level of oestrogen in this organ, plus slowing down its metabolic disposition that would prolong its half-life in the pancreas. These effects are indeed observed in the present study using the oestrogen receptor-positive pancreatic cancer cells that selectively overexpress PDIp. Taken together, the oestrogen enrichment and protective effects of PDIp would favour pancreatic cells to have an oestrogen-rich microenvironment. As such, PDIp may enhance the regulatory effect of oestrogens on...
certain pancreatic normal functions, such as zymogen production and insulin secretion [10–12,15–17]. In addition, it is also tempting to suggest that PDIp is probably a factor that may augment the presumed protective effect of oestrogen against pancreatic tumorigenesis [18–20,24] and against islet cell death [12]. Undoubtedly, the exact role of PDIp in modulating the biological actions of oestrogen needs further investigation in the future.

Lastly, it is of considerable interest to note that it was reported approximately two decades ago that the pancreas expresses a quantitatively major unique E2-binding protein, although the nature of this oestrogen-binding protein is still not clear to this day [39,40]. This protein was reported to be specifically expressed in normal pancreatic acinar cells with a dominant subcellular localization in the endoplasmic reticulum, but undetectable in pancreatic tumour cells [39,40]. It is postulated that PDIp might be the previously unidentified E2-binding protein in the pancreas, on the basis of the observations from previous studies and particularly the observations made in the present study. First, PDIp is specifically and highly expressed in pancreatic acinar cells [25,28,31]. Secondly, PDIp contains a signal peptide that guides the protein to localize at endoplasmic reticulum [28]. Thirdly, PDIp expression is dramatically reduced or even undetectable in human pancreatic tumour tissues compared with normal pancreatic tissues [28]. Fourthly, PDIp has an E2-binding activity that accounts for approximately 80% of the total E2-binding activity in the pancreas.

AUTHOR CONTRIBUTION
Xinxiao Fu designed and performed most of the experiments, analysed the results and wrote the paper, Pan Wang cultured cells, Chong Long and Lixin Wang prepared animal tissues and detected PDIp protein levels; Hye Joung Choi and Masayuki Fukui measured the oestrogen levels in animal tissues; and Bao Ting Zhu participated in performing some of the experiments, performed data analysis, and wrote and finalized the paper.

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SUPPLEMENTARY ONLINE DATA

PDIp is a major intracellular oestrogen-storage protein that modulates tissue levels of oestrogen in the pancreas

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Figure S1  Time-dependent change in uterine wet mass in overiectomized female rats following administration of E2

The administration of E2 is described in the Experimental section in the main text.

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Figure S2 Isolation of natural PDIp from rat and human pancreatic tissues by SEC and IEC

(A) SEC isolation of PDIp from rat (blue curve) and human (red curve) pancreatic tissues. Human pancreatic tissue lysate was pre-treated as described in the Experimental section of the main text and then loaded on to the Superdex 200 column for SEC separation (eluted with 10 mM sodium phosphate buffer, pH 7.4). The supernatant of rat tissue lysate was directly loaded on to the column for SEC separation. (B) IEC isolation of the rat PDIp from fractions A7 and A8 in (A). The A7 and A8 fractions from four SEC separation experiments using rat pancreas were combined, centrifuged and then loaded on to Mono Q for IEC (eluted with a NaCl gradient in 10 mM sodium phosphate buffer, pH 7.4). The gradient NaCl elution was performed as follows: (i) increase from 0 M to 0.15 M NaCl in 1 column vol.; (ii) increase to 0.4 M NaCl in 12 column vol.; and (iii) increase to 1 M NaCl in 2 column vol. and then back to 0 M NaCl. (C) Isolation of human PDIp from the A7 and A8 fractions in (A) by IEC. Gradient NaCl elution was performed as follows: (i) increase from 0 M NaCl to 0.15 M NaCl in 1 column vol.; (ii) increase to 0.35 M NaCl in 12 column vol.; and (iii) increase to 1 M NaCl in 2 column vol. and then back to 0 M NaCl.

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Figure S3 Isolation of human PDIp by IEC

(A) IEC isolation of PDIp using different elution conditions as described for Figure S2(C). The gradient NaCl elution was performed as follows: (i) increase from 0 M to 0.15 M NaCl in 1 column vol.; (ii) increase to 0.4 M NaCl in 12 column vol.; and (iii) increase to 1 M NaCl in 2 column vol. and then back to 0 M NaCl. (B) Protein level (left-hand y-axis) and bound [3H]E2 (right-hand y-axis) of the fractions collected in (A). Each fraction was subjected to determination of protein concentration and [3H]E2-binding activity. (C) Western blot analysis of ERα, ERβ, PDI and PDIp in A9, A10 and A11 fractions from (A).