SILYMARIN INDUCES GLUCAGON LIKE PEPTIDE-1 SECRETION AND INCREASES ITS RECEPTOR GENE EXPRESSION IN PANCREATIC TISSUE IN PARTIALLY PANCREATECTOMIZED RATS

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Abstract-
Glucagon-like peptide-1 (GLP-1) is an incretin produced and released by gut endocrine L-cells in response to the ingestion of carbohydrates, lipids and proteins. It is also produced in the pancreatic tissue, both in humans and rats. In the pancreatic tissue, GLP-1 stimulates insulin secretion in β-cells, induces neogenesis, proliferation, differentiation and maintenance of β-cell mass, and inhibits apoptosis of these cells. For these reasons, this peptide has been used in the management of type 2 diabetes mellitus (DM) and has also been tried in type 1 DM. The action of GLP-1 on β cells is initiated through binding with a GLP-1 membrane bound receptor (GLP-1R), coupled to G protein, which leads to a rise in cyclic AMP (cAMP) and calcium levels, followed by insulin secretion. It was reported that GLP-1 induces an increase of PDX-1 gene expression levels and enhances the transcription of the insulin gene by increasing the binding of PDX-1 transcription factor to the insulin gene promoter region. We reported previously that silymarin induces both PDX-1 transcription factor and the insulin gene expression, as well as β-cell proliferation in pancreatic tissue. In this study we demonstrated that silymarin treatment to partially pancreatectomized rats increases pancreatic GLP-1R gene expression, tissue immunoreactivity for GLP-1, densitometry of this peptide for Western blot analysis, proliferation of GLP-1 secreting cells, insulin serum concentration and a decrease in glucose serum. All these tests were performed to show the contrast with untreated pancreatectomized animals. Our results may suggest that silymarin increases the secretion of pancreatic GLP-1, and its activity. It would seem that it may be related to the increase in the PDX-1 transcription factor previously reported in silymarin treatment of partially pancreatectomized rats.

Keywords—proliferation of pancreatic GLP-1 producing cells, GLP-1 pancreatic receptor, GLP-1R, GLP-1 pancreatic immunoreactivity

I. INTRODUCTION
Glucagon-like peptide-1 (GLP-1), is produced and released by gut endocrine L-cells located predominantly in the distal small intestine and colon, in response to the ingestion of carbohydrates, lipids and proteins [1]. Some authors have reported that GLP-1 may also be produced in the pancreatic islets, both in human [2,3] and rat pancreas [4] residing in α-cells [5]. GLP-1 is a product of post-translational cleavage of pro-glucagon peptide [6, 7]. At the pancreatic level, GLP-1 stimulates insulin secretion in β-cells in a dependent blood glucose concentration [8], induces differentiation, neogenesis, proliferation, and plays a role in the long-term maintenance of β-cell mass by inhibiting apoptosis of these cells, both in human and in animal models [9, 10]. GLP-1 also suppresses α-cell glucagon secretion, increases δ-cell somatostatin, delays gastric emptying and reduces appetite [11]. For these reasons, therapies based on this peptide have been used in the management of type 2 diabetes mellitus [12, 13] and in some studies it has been reported that this treatment has been tried on type 1 DM [13]. The action of GLP-1 on β-cells is initiated through binding to the GLP-1R membrane bound receptor, which is coupled to G protein [14, 15]. The activation of GLP-1R leads to a rise in cyclic AMP (cAMP) and calcium levels followed by insulin secretion. In this process, GLP-1R enhances the inhibition of ATP-sensitive potassium channels (K+ATP) [16] and facilitates the Ca2+ influx and stimulation of insulin exocytosis [17]. The principal effectors of this cAMP mechanism are creatin phosphokinase A, (PKA) and exchange protein directly activated by cAMP2 (EPAC2). GLP-1 both in vitro and in vivo enhances insulin gene transcription by the increase of the binding PDX-1 transcription factor to the insulin gene promoter region [18, 19, 20, 21]. Pancreatic duodenal homeobox 1 (PDX-1) is considered to be the key transcription factor involved in early pancreatic development, the expression, differentiation and maintenance of the mature β-cells [22]. PDX-1 is a key regulator of insulin gene expression. Also by cAMP response element-binding protein (CREB) which after activating GLP-1R binds to cAMP responsive element (CRE) localized in the insulin gene promoter region [23]. All these actions contribute to β-cell mass. However, it was reported that the effect of GLP-1 on pdx-1 promoter region activity is short lived and that only
Animals were anesthetized for sacrifice with sodium pentobarbital (50 mg/Kg b.w., i.p.). Blood was obtained
through cardiac puncture. The pancreas was extracted by dissecting the abdominal cavity and removing fat tissue.

C. RNA isolation and RT–PCR assay for GLP-1R
RNA was isolated from a fragment of the pancreatic tissue (50 mg) of each animal under the different experimental
conditions described above and was prepared using the column method according to the manufacturer's protocol
PROMEGA (Madison, WI). Briefly, the tissue was homogenized in 1000 µL of lysis buffer. 350 µL of dilution
buffer was added to 175 µL of lysate. The sample was heated at 70°C for 3 minutes and centrifuged for 10 minutes. 250 µL
of 95% ethanol was added to clear lysate and mixed. The lysate was transferred to column and centrifuged for 1
minute. 600 µL of wash solution was added and centrifuged for 1 minute. 50 µL of DNase was added to column and
incubated for 15 minutes at room temperature. After this time, 200 µL of DNase stop solution was added and
centrifuged for 1 minute and washed twice. To elute the RNA, 100 µL of nuclease free water was added to membrane.
RT-PCR was performed with 2 µg of RNA for the target gene and the 18s ribosomal constitutive gene for semi-quantitation.

II. METHODS
Silymarin and all reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Promega (Madison,
WIS), Bio Rad (Bio Rad Laboratories Inc.) or from local suppliers (Merck and J.T. Baker, México) and were of
analytical grade. Testing and experiments performed in this study followed the guidelines stated in "Principles of
Laboratory Animal Care" (NIH publication #85-23, revised 1985) and Mexican regulations "Norma Oficial Mexicana
 NOM-062-ZOO-1999, Especificaciones técnicas para la produccion, cuidado y uso de animales de laboratorio"
(published in December, 1999). The studies were approved by the committee of ethics of the Biological Sciences
Division Council in our University.

A. Animal treatment
Male Wistar rats (200-220 g of b. wt.) were obtained from our animal facility, fed with Purina standard chow, and
maintained at 20-22°C with 12-h cycles of light/dark. The animals were divided into the following groups: 1.-
Pancreatectomy group: 36 rats were anesthetized with ketamine (50 mg/Kg) and xylazine (5 mg/Kg). 60% of pancreatic
tissue was removed by cotton applicators rubbed gently. These animals in turn were divided into 6 groups for
the following sacrifice times: 3, 7, 14, 21, 42 and 63 days after pancreatectomy. Three animals from each group
were administered with 5-bromo-2'-deoxyuridine (BrdU) (50 mg/Kg b.w. i.p.)., 18 hours before sacrifice to assess the
proliferation of GLP-1 secreting cells. Their body weight and serum glucose were measured weekly for each fasted rat. 2.-
Pancreatectomy + silymarin: The same schedule was followed as with group 1. Pancreatectomized animals were
administered with silymarin daily (200 mg/Kg b.w. p.o.) n=36. 3.- Control: These animals underwent a sham surgery
and were sacrificed before treatments began (n=6). Each week, animals were fasted for 7 hours. Blood glucose was
measured with an automatic glucometer (Abbot Diabetes Care Ltd. U.K.) in the tail vein and its weight was registered.

B. Blood and tissue collection
Animals were anesthetized for sacrifice with sodium pentobarbital (50 mg/Kg b.w., i.p.). Blood was obtained
stimulates this activity for about 2-3 hours. In a previous report, we showed that silymarin induces both PDX-1 and the
insulin gene expression as well as proliferation of β-cells in pancreatic tissue [24]. This study shows that silymarin treatment to partially
pancreatectomized rats increases pancreatic GLP-1R expression, GLP-1 tissue immunoreactivity, densitometry of this
peptide for Western blot analysis, proliferation of GLP-1 secreting cells, insulin serum concentration and a decrease in


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immunoreaction, these sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min and were immunolabelled with mAbs anti-BrdU (1:100) at room temperature for 2 hours. HRP-goat anti-mouse IgG (1:50) was added for 90 min. at room temperature. A mixture of NiCl<sub>2</sub> (1%) + CoCl<sub>2</sub> (1%) was added to DAB/ H<sub>2</sub>O<sub>2</sub> solution for color development for 8 min. Then sections were counterstained with hematoxylin. Double immunolabel was seen in black color. Sections were analyzed with a 40X objective. The total number of cells and double immunolabelled cells were counted in five tissue areas with a Leica Application Suite program on a Leica DM 1000 microscope.

F. Pancreatic tissue GLP-1, glucagon double immunoassay

In order to prove GLP-1 secretion in α-pancreatic cells, a glucagon, GLP-1 double immunoassay was made for the first double immunolabel as described above (2.5 section). mAbs anti-GLP-1 (1:100) was utilized as a primary antibody and HRP-rabbit anti-goat IgG (1:75) as a secondary antibody. As a primary antibody mAbs anti-glucagon (1:75) was used for double immunostaining α-pancreatic cells and HRP- goat antirabbit IgG (1:100) as a secondary antibody. Double-immunolabelled sections were analyzed with a DM-1000 Leica microscope.

G. Western blot analysis

To determine the level of pancreatic GLP-1, a fragment (40 mg) of this tissue was homogenized in 10 mL of an ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine, 1 mg/mL leupeptin, and 1 mg/mL phenylmethylsulfonyl fluoride, pH 7.6) at 15,000 rpm with 3 strokes per 15 sec with a tissue homogenizer (IKA Works, Inc. Wilmington, NC). After homogenization, total protein concentrations were measured using the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA). Samples were stabilized by adding 1 vol of 2X Laemml sample buffer to 1 vol of sample, and heated at 70°C for 10 min. 100 µg of protein from each sample were loaded into individual lanes and electrophoresed on 12.5% polyacrylamide-SDS minigels using a Mini-PROTEAN Tetra Cell electrophoresis apparatus (Bio-Rad Laboratories Inc.). The proteins were then transferred electrophoretically to PVDF membrane (Immobilon–P, Millipore, Billerica, MA, USA). After blocking with 5% skim milk in PBS-T (80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, and 0.2% Tween-20, [pH7.5]) for 1 hour, the membranes were probed overnight with mAbs anti-GLP-1 as a primary antibody at 4°C (1:500) (Zymed Labs, Inc, San Francisco) and then incubated with secondary horseradish peroxidase conjugated antibody (1:1000) (Zymed Labs, Inc, San Francisco). Antigen-antibody reactions were viewed using enhanced chemiluminescence substrate (Immobilon Western, Millipore, Billerica, MA, USA) and analyzed in Chemidoc XRS (Bio-Rad, Laboratories, Inc) with the Quantity One 1-D Analysis v4.6.5 image processing software (Bio-Rad).

H. Statistical methods

ANOVA followed by Tukey and Dunnet tests were used to compare experimental and control groups (SPSS, Chicago, IL, USA) with significance at p < 0.05.
Figure 2. Proliferation of GLP-1 secreting cells. These were counted as percentage of total cells in each field of five fields in each animal pancreatic tissue at different times after pancreatectomy: (A) untreated; (B) silymarin treated; (C) control group. Each bar represents the mean ± S.E.M. * p < 0.05 versus other times of the same group. + p < 0.05 treated versus untreated animals at the same times. n = 5.

Figure 3. Double immunolabelling for GLP-1 and BrdU. Photomicrographs of double immunolabelled cells of pancreatic tissue (arrows) (×400 magnification). Scale bar = 25 μm. (A) untreated group; (B) group treated with silymarin. Both at 63 days after pancreatectomy; (C) control group.

C. GLP-1, glucagon double immunolabelled pancreatic tissue cells

These double immunolabels for glucagon and GLP-1 in the pancreatic tissue of some experimental groups are shown in Figure 4. These photomicrographs indicate that this GLP-1 peptide is produced in α-pancreatic cells, as has been reported. These double immunolabels were not counted but it may be appreciate a greater number of double immunolabelled cells in the treated groups.

D. GLP-1 Western blot analysis
This analysis shows GLP-1 pancreatic peptide at 2 and 9 weeks of the treatment. The level of this peptide was increased in the groups of pancreatectomized animals treated with silymarin. This increase was greater in the group treated for nine weeks than in the other groups (Figure 5).

Figure 4. Double immunolabelling for GLP-1 and glucagon. Photomicrographs of double immunolabelled cells of pancreatic tissue (arrows) (×400 magnification). Scale bar = 25 μm. (A) untreated group; (B) group treated with silymarin. Both at 63 days after pancreatectomy; (C) control group.

Figure 5. Representative Western blot analysis of pancreatic GLP-1 peptide and β-actin of partially pancreatectomized rats. (A, C) untreated group and (B, D) group treated with silymarin. Where (A, B) is at 14 days and (C, D) is at 63 days after pancreatectomy.

E. Serum insulin and glucose levels
Serum insulin levels are shown in Table 1, showing a variation in values in respect to the control group (1.15 ±
0.02 mM/L). These values were higher in the groups treated with silymarin (except day 7). Table 2 shows glucose serum levels. At all times after pancreatectomy, the serum glucose levels of untreated animal groups were maintained over the control value group (5.7 ± 0.005 mM/L). Silymarin treatment produced a significant decrease of serum glucose levels in respect to the untreated group for the same time after pancreatectomy. These results could be related to the insulin serum levels of the silymarin treated groups.

Table 1. Serum insulin levels (ng/mL) in experimental groups

<table>
<thead>
<tr>
<th>Days after pancreatectomy</th>
<th>Untreated group</th>
<th>Silymarin treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.15 ± 0.02</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.550 ± 0.070*</td>
<td>0.677 ± 0.125</td>
</tr>
<tr>
<td>7</td>
<td>1.18 ± 0.45</td>
<td>0.006 ± 0.023</td>
</tr>
<tr>
<td>14</td>
<td>0.333 ± 0.037*</td>
<td>0.875 ± 0.322*</td>
</tr>
<tr>
<td>21</td>
<td>0.18 ± 0.08*</td>
<td>0.84 ± 0.121</td>
</tr>
<tr>
<td>42</td>
<td>1.20 ± 0.222</td>
<td>2.51 ± 0.222 *</td>
</tr>
<tr>
<td>63</td>
<td>1.143 ± 0.37</td>
<td>3.245 ± 0.255*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. * p < 0.05 versus other times of the same group; + p < 0.05 treated versus untreated animals at the same times. n = 5.

Table 2. Serum glucose levels mM/L in experimental groups

<table>
<thead>
<tr>
<th>Days after pancreatectomy</th>
<th>Untreated group</th>
<th>Silymarin treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7 ± 0.002</td>
<td>5.7 ± 0.002</td>
</tr>
<tr>
<td>3</td>
<td>0.55 ± 0.055</td>
<td>0.677 ± 0.125</td>
</tr>
<tr>
<td>7</td>
<td>0.80 ± 0.000</td>
<td>0.5 ± 0.141*</td>
</tr>
<tr>
<td>14</td>
<td>0.20 ± 0.065</td>
<td>7.9 ± 0.252*</td>
</tr>
<tr>
<td>21</td>
<td>7.74 ± 0.200</td>
<td>8.00 ± 1.50</td>
</tr>
<tr>
<td>42</td>
<td>10.65 ± 0.065</td>
<td>7.15 ± 0.266*</td>
</tr>
<tr>
<td>63</td>
<td>8.54 ± 0.006</td>
<td>0.20 ± 0.155*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. + p < 0.05 treated versus untreated animals at the same times. n = 5.

IV. DISCUSSION

It is well documented that some of the more important GLP-1 effects are the induction of β-cell neogenesis [26]. This peptide plays an important role for the long-term maintenance of β cell mass, which contributes 80% to glucose homeostasis [27]. It has been proposed that the majority of GLP-1 effects are transduced by a single GLP-1 receptor, cloned from pancreatic β-cells [15]. De León et al. [28] reported that in CD-1 mice with inactivated gene of GLP-1R (glp-1R-/-) after pancreatectomy, showed a significant defect in β-cell mass regeneration. Yazhou et al. [29] reported in isolated rat β-cells that GLP-1 receptor signaling reduced apoptosis induced by cytokines. These findings demonstrate the importance of GLP-1 receptor for activating this peptide. Our results show that silymarin treatment significantly increased the gene pancreatic expression of this receptor, which may increase the binding of GLP-1 with its receptor and therefore the actions of the peptide. Despite the short-time action of GLP-1, there are some therapies based on the use of GLP-1 in the management of type 2 diabetes mellitus [12, 1], as exendin-4, which is an agonist of GLP-1 and is active for a longer period of time. In a model of partial pancreatectomy, the administration of exendin-4 for 10 days after surgery produced a stimulation of pancreatic regeneration, and an expansion of β-cell mass by the neogenesis and proliferation of these cells with attenuated pancreatectomy hyperglycemia [30]. This author showed that exendin-4, stimulates both the differentiation of β cells from ductal progenitor cells (neogenesis) and proliferation of these β cells in a partial pancreatectomy rat model of type 2 diabetes mellitus, inducing the regeneration of the pancreas and β cell mass.

In this study we demonstrated that, in addition to the increase of GLP-1R gene expression induced by silymarin, the treatment with this compound to partially pancreatectomized rats, induced an increase of immunoreactivity for GLP-1 in pancreatic tissue (Figure 3), an enhanced pancreatic tissue level of this peptide (Figure 5), an increase in the proliferation of pancreatic GLP-1 secreting cells (Figure 2), an increase in insulin blood levels (Table 1) and therefore, a decrease in blood glucose levels (Table 2). The effects reported for this peptide are in accordance with those in the bibliography and were higher in treated than in untreated animals. This may suggests that the increase in these effects may be related to the silymarin treatment on the GLP-1 secretion, since there was an increase in the results observed. Wideman et al. [31] showed that by inducing GLP-1 secretion directly within pancreatic islets by means of PC1/3 (prohormone convertase that produces GLP-1 from α-cell glucagon) through adenosine-mediated expression in these cells, increases GLP-1 secretion, improving glucose-stimulated insulin secretion, possibly by enhancing nuclear PDX-1 transcription factor and insulin content of islet β cells. It was reported that the GLP-1 analog, exendin-4, enhances the pancreatic expression of PDX-1 transcription factor, which plays a critical role in pancreas development, differentiation, maintenance of β pancreatic cells and plays a key role in insulin gene expression [32], thereby stimulating β cell neogenesis and increasing islet size [21]. Campbell et al. [33] examined the effect of GLP-1 on pdx- promoter activity in the cell line Min 6. When these cells were stimulated with GLP-1 (10 nM) at low concentrations of glucose, an increase in transcriptional activity was observed with the full length pdx-1 promoter region compared to low glucose alone. When the pdx promoter region was stimulated with GLP-1 at high glucose concentrations, there was a further increase in transcriptional activity, which evidenced that GLP-1 positively regulates the promoter region of pdx-1 gene activity and stimulates the mRNA levels of PDX-1, increasing the PDX-1 protein both in vitro and in vivo [34, 35, 36]. This may induce an increase in binding the PDX-1 protein to the insulin gene promoter region. PDX-1 regulates the insulin gene expression [34, 37] and causes an increase in insulin mRNA levels. This could explain the GLP-1 pancreatic activity in the induction of neogenesis and maintenance of β cell mass. We previously reported that silymarin increased the gene expression of the transcription
factor PDX-1, its tissular immunoreactivity and β cell proliferation [24]. The previously described findings in this section may suggest that silymarin induces the increase of GLP-1 secretion and its effects. The results obtained in this study suggest that it may involve an increase in the gene expression of the transcription factor PDX-1, its tissular immunoreactivity, β pancreatic cell proliferation and an increase in blood insulin levels [24]. Through the analysis of mice with specifically inactivated pdx-1 gene in β-cells (β-celldpx1-/- mice), Yazhou et al. [38] demonstrated that PDX-1 is essential in β-cells for the regulatory, proliferative and cytotoxic actions of GLP-1. Studies by these authors demonstrate that endoxin-4 induces a decrease in glucose blood levels after a glucose challenge, an increase in plasma insulin, pancreatic insulin content and glucose-stimulated insulin secretion in islets of mice with β cells pdx-1+/+ but not in mice pdx-1-/-.. Also, endoxin-4 significantly increased β cell proliferation, reduced apoptosis and increase the levels of insulin mRNA transcripts in mice with β cells pdx-1+/+ but not in mice with pdx-1-/-.. These findings demonstrate that PDX-1 gene expression is essential for the integration of GLP-1 dependent signals for the GLP-1 secretion from α cells and for the activity of this peptide on β cells. In turn, GLP-1 enhances the pancreatic gene expression of PDX-1, which overall may suggest a necessary coordination between GLP-1, GLP-1R and PDX-1 for correct activity of GLP-1.

Furthermore, Loffy et al. [26] showed that GLP-1 treatment of normal and diabetic rats significantly increases the pancreatic antioxidant enzymes, catalase and glutathione reductase, which contribute in protecting β cells from free radicals that might kill the cells since they are particularly susceptible to attack by free radicals. We also reported [39] that silymarin increases the effect and activity of pancreatic antioxidant enzymes: catalase, glutathione peroxidase and superoxide dismutase. We may suggest by the Loffy et al. [26] studies that the increase of expression and activity of these enzymes were induced by GLP-1, which as we have pointed out is increased by silymarin treatment and therefore could prevent the death of β cells.

V. CONCLUSION

In conclusion, the results of our study indicated that the application of the silymarin treatment induced an increase of GLP-1R gene expression and GLP-1 secretion in pancreatic tissue. Since this peptide enhances the PDX-1 pancreatic expression and its transcription factor’s activity, observed in our previous studies with this compound [24], this indicates that silymarin should be considered a compound potentially useful in the treatment of diabetes mellitus.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest

REFERENCES


