Glutamninase activity is confined to the mantle of the islets of Langerhans

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Abbreviations: KGA, kidney-type glutaminase; LGA, liver-type glutaminase; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate

Abstract

Glutamatergic signalling plays an important role in the coordination of hormone secretion from the endocrine pancreas. Thus, glutamate production must be a process exquisitely regulated to ensure a proper transmitter function. Recently we have reported that the endocrine pancreas co-expresses two isoforms of the protein glutaminase (GA), denoted as kidney-type (KGA) and liver-type (LGA). However, how GA activity, and therefore glutamate production, is regulated in the islets represents a critical issue that remains unresolved. Since the purification of these enzymes from rat islets is a daunting task, in order to characterize each isoform we have taken advantage of the spatial segregation of these isoenzymes in pancreas. To assist us with this goal, we have developed a new procedure that enables us to assay GA activity in situ. The assay is highly specific for GA as indicated by its dependence on glutamine and orthophosphate. Surprisingly, LGA, which is abundantly expressed by beta-cells, did not show detectable activity under the assay conditions. All the GA activity detected in pancreatic islets was attributed to KGA and was confined to the mantle of the islets. Double labelling analyses strongly suggested that alpha-cells should be regarded as the site of glutamate production in the endocrine pancreas.

1. Introduction

Insulin and glucagon, two of the main hormones involved in glucose homeostasis, have opposite effects on target tissues. Not surprisingly, their secretion from the endocrine pancreas must be tightly coordinated. In this sense, the glutamatergic signalling system has been proved to be present in islets of Langerhans where it can affect hormone secretion [1], [2]. Indeed, the endocrine pancreas expresses both ionotropic and metabotropic glutamate receptors, which have been shown to be functional [3], [4], [5].

Therefore, glutamate is postulated as an autocrine and paracrine-signal enabling alpha and beta cells to communicate within the islets [6], [7]. Although the importance of glutamate as an extracellular transmitter in the islets of Langerhans seems to be beyond discussion, a number of conflicting results do not allow to correlate consistently the activation of glutamate receptors with either insulin or glucagon secretion [8]. These observations stress the complexity of glutamate-mediated signalling, as well as underscore the importance of gaining a better understanding of where and how the glutamate signal is produced within the islets.

Since glutamate does not readily enter the islets, it must be generated internally [9]. Glutamine, an abundant and signalling inert amino acid, is an obvious candidate as the precursor of the transmitter glutamate. Recently, we have noticed that pancreas co-expresses two isoforms of glutaminase (GA; EC 3.5.1.2), denoted as kidney-type (KGA) and liver-type (LGA) [10]. Furthermore, in immunofluorescence labelling and laser-scanning confocal microscopy studies, these enzymes showed a striking complementary pattern of cellular expression. Whereas KGA was mainly present in α-cells, LGA was very abundant in β-cells [11]. Because β-cells account for the bulk of the endocrine cell population, we concluded that LGA was the major GA protein in pancreas. However, an inherent limitation of the technique used to derive this conclusion is that it reveals the amount of enzyme protein, rather than the level of enzyme activity. In order to overcome this limitation, we have developed, using unfixed cryostat sections and specific GA activity staining, a new method that allowed us to discriminate *in situ* the relative activities of KGA and LGA. In the current study, we describe the procedure as well as the surprising findings obtained through its use. The functional relevance of such findings will also be discussed in the context of the glutamatergic communication and its role in the regulation of hormone secretion.

2. Material and methods

2.1. Animals and tissue processing

Male Wistar rats (225–250 g) were used in these studies. The animals were sacrificed by decapitation. Pancreas was removed immediately after sacrifice. Small fragments of the tissue were frozen in liquid-nitrogen-cooled isopentane and embedded in Tissue-Tek® OCT™ compound for cryostat sectioning. Cryostat sections, ranging from 12 to 40 μm thick, were cut on a cryostat at a cabinet temperature of −25 °C. Sections were picked up onto polylysine-coated glass slides and immediately submitted to GA activity histochemical staining. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the committee of animal use for research at Málaga University. All efforts were made to minimize the number of animals used and their suffering.

2.2. Activity assay

GA activity was demonstrated using a tetrazolium salt method. Unfixed cryostat sections were allowed to adjust to room temperature. Afterwards, 100 µl of medium were poured onto sections which were then incubated in darkness for 30 min at 37 °C. The incubation medium contained: 5% w/v polyvinyl alcohol (average M_r31,000–50,000) as tissue protectant; 200 mM Tris–HCl buffer (pH 8.0); 200 mM KH_2PO_4; 40 mM glutamine; 0.1 mM EDTA; 3.4 mM NAD^+; 0.5 mM ADP; 0.3 mM nitroblue tetrazolium (NBT); 0.49 mM phenazine methosulfate (PMS); and 100 units of glutamate dehydrogenase (GDH, EC. 1.4.1.2) used as an auxiliary enzyme. As described above, the medium for test reaction contained both glutamine (the GA substrate) and phosphate (an essential activator of GA). However, in the medium used for control reactions either glutamine or phosphate was omitted. After incubation the sections were treated as described elsewhere [12]. Briefly, sections were washed with 100 mM phosphate buffer pH 5.3 (to stop the reaction immediately) at 60 °C, (to remove the incubation medium rapidly), postfixed in 4% formaldehyde and mounted in glycerol medium.

2.3. Immunocytochemistry and double-labelling

Serial sections were submitted to GA activity assays and immunodetection of glucagon-containing cells, respectively. Sections for immunocytochemistry were fixed in 4% paraformaldehyde during 30 min. After three washes and blocking the endogenous peroxidase activity, as well as blocking endogenous avidin, biotin and biotin-binding proteins, sections were incubated overnight at 4 °C with a mouse monoclonal anti-glucagon antibody (diluted 1:10,000, Sigma), and processed by the avidin–biotin method using biotinylated anti-mouse IgG (1:1000, Dako) and ExtrAvidin–peroxidase conjugate (1:2000, Sigma). Immunoreaction product was visualized with 0.05% diaminobenzidine, 0.03% nickel ammonium sulphate, and 0.01% H_2O_2. For the double-labelling experiments, the procedure was as follows. After having carried out the GA activity staining and washed the sections as described above, the sections were fixed for 30 min in 4% paraformaldehyde, washed and incubated overnight at room temperature with a primary antibody. Primary antibodies were raised against glucagon (mouse monoclonal antibody, diluted 1:2000; Sigma), insulin (mouse monoclonal antibody, diluted 1:2000; Sigma) and somatostatin (goat polyclonal antibody, diluted 1:1000; Santa Cruz Biotechnology). After washing three times with PBS, sections were incubated during 90 min with the secondary antibody: Alexa Fluor 568 goat anti-mouse (1:1000, Molecular Probes) or donkey anti-goat (1:1000, Molecular Probes).

3. Results

Although LGA was originally believed to be present only in liver, a growing number of studies have now reported the co-expression of LGA and KGA in different cells and tissues. Nevertheless, only the LGA enzyme from rat liver has been kinetically characterized so far. The characterization of extrahepatic LGA has been hampered by the difficulty in isolating LGA from KGA throughout the purification procedures. In a recent work, we have reported a remarkable spatial segregation of KGA and LGA in rat islets [11]. Thus, we have reasoned in the following way. If we were able to assay GA activity *in situ*, then it would be possible to dissect the catalytic properties of each pancreatic GA isoenzyme. To develop such a method we have modified, and adapted for unfixed tissue sections, a GA assay that was originally designed to detect GA activity on native PAGE [13]. The enzyme was specifically revealed by incubation of unfixed cryostat sections with glutamine and coupling the oxidation of the glutamate formed to the reduction of a tetrazolium dye, resulting in precipitation of the insoluble and coloured formazan. This is achieved with the help of exogenously added glutamate dehydrogenase which acts as an auxiliary enzyme.

The standardized activity staining procedure was applied to pancreas sections ranging in thickness from 12 to 40 μm. While thicker sections showed more activity, higher morphological resolution was achieved with lower GA activity. In other words, when formazan was over-accumulated it made difficult to discriminate positive and negative neighbouring cells (not shown). Finally, we concluded that 14 μm thick sections and 30 min incubation at 37 °C yielded the best results. Fig. 1A shows the localization of formazan precipitation in a pancreas section at low magnification. Beside blood vessels and adipose tissue, formazan production was mainly found in spheroid cell clusters dispersed throughout the exocrine parenchyma. That is, the staining was mainly observed in structures resembling islets of Langerhans. In order to confirm that such structures are indeed pancreatic islets, an antibody against glucagon was used as a marker of α-cells. Thus, serial sections were submitted to GA activity assay and immunocytochemistry, respectively. The results confirmed that formazan stained islets of Langerhans (Fig. 1, compare B1 with B2).

Since endogenous dehydrogenases, including GDH, occur in pancreatic cells, the specificity of the staining observed in islets needed to be addressed. If the accumulation of formazan was a consequence of the GA reaction, the staining should disappear when glutamine, the substrate of the GA reaction, is removed. That was precisely what happened (Fig. 1B3). Moreover, the effect of removing exogenous phosphate from the incubation medium was tested. Inorganic phosphate is an essential activator of GA [14]. Accordingly, if the staining we observed in pancreas sections was a reflection of the presence of GA activity, that staining should be sensitive to the levels of the inorganic anion phosphate. Fig. 1B4 confirms that this was the case. It must be noted that in the phosphate-omitted controls, although the staining was drastically reduced, a weak albeit detectable signal was observed. This result is congruent with the finding that in the nominal absence of phosphate, the concentration of endogenous phosphate was around 10 mM [15]. Overall, these observations strongly suggest that the histochemical staining observed in pancreas was a consequence of the presence of an active GA enzyme in this tissue.

An unexpected finding was that GA-specific staining was confined to the mantle of the islets (Fig. 1). Furthermore, in serial sections, the comparison between the areas showing GA activity (Fig. 1B1) and those which are glucagon immunopositive (Fig. 1B2) suggested that GA activity was confined into glucagon-containing α-cells. To further address this question, we next carried out double-labelling on the same cryostat sections, using histochemical GA activity staining and immunofluorescent labelling for glucagon, insulin and somatostatin. Fig. 2 supports the co-localization of GA activity and glucagon into the same cells.

Because different isoenzymes may exhibit different requirements, and, unlike KGA, an active LGA could not be detected in pancreas, we next addressed the suitability of our activity assay for LGA. To this end we used rat liver as a source of LGA enzyme. Using the redox-cycling reaction described herein, we managed to detect phosphate-dependent glutaminase activity both in crude liver extracts and liver sections (Fig. 3). For comparative purposes, kidney sections, lacking LGA but containing KGA, processed for GA activity are also shown in this figure.

4. Discussion

Glutamine, the most abundant free amino acid in the body, has a broad range of metabolic functions [16]. Recently, it has been suggested that glutamine-derived glutamate may act as a transmitter in non-neuronal tissues such as bone, testis and pancreas [17]. In all these cases, GA would catalyse the hydrolytic cleavage of glutamine to form glutamate and ammonium. Despite the variety and importance of the cellular functions where GA is involved, our knowledge regarding the tissue-specific regulation of GA is largely restricted to the enzymes found in kidney and liver. The enzyme from liver, unlike the kidney-type, is not inhibited by glutamate and shows less dependence on phosphate [14]. Mammals express at least three different GA isoenzymes that are derived from two related but distinct genes designed as liver and kidney types [10]. The liver-type gene encodes a protein referred to as LGA, while the kidney-type gene directs the transcription of two splicing variants named KGA and GAC (see [18] for a recent and concise review).

The LGA isoenzyme was originally thought to be present only in adult liver [14]. However, a growing number of studies have now clearly demonstrated that expression also occurs in extrahepatic tissues such as brain [19], pancreas [11] and cancer cells of diverse origins [20]. In all these cases LGA was co-expressed together with either KGA or GAC. An intriguing observation, common to all these cases without exception, was the failure to prove that a fraction of the GA activity measured in extracts exhibited properties characteristic of the hepatic enzyme. This fact is especially remarkable in the endocrine pancreas. Whereas the kinetic properties of islets GA activity suggest that it is a kidney-type enzyme [15], at the protein level KGA only represents a small fraction of the total GA present in the islets [11]. To investigate these issues, we have first developed a new method that allows the detection of GA activity in tissue sections. Second, we have taken advantage of the spatial segregation that both isoforms, KGA and LGA, exhibit in islets of Langerhans. Thus, using *in situ* GA activity staining, we have concluded that the enzyme is only detectable, under the assay conditions used, in peripheral islet cells (Fig. 1). Moreover, using insulin as a marker of insulin-secreting β-cells, double labelling experiments suggested that no peripheral β-cells, and therefore no LGA, were contributing to the detected GA activity. Since these two isoenzymes exhibit different requirements, we cannot rule out the possibility that the current assay underestimates the activity contributed by LGA.

The current study raises the intriguing question of what are the particular properties of β-cell LGA, which preclude its detection under the assay conditions. It is interesting to note that glutamine can be synthesized by glutamine synthetase (GS; EC 6.3.1.2) in β-cells, where glutamine can affect glucose-stimulated insulin release without being metabolized via glutamate [21]. On the other hand, there is a body of evidence indicating that glutaminolysis plays an important role in basal and amino acid stimulated insulin secretion [15], [22], [23]. Therefore, in β-cells, which express an active GS [21], an unregulated constitutively active LGA may lead to a futile substrate cycle, consuming ATP and preventing the signalling role postulated for glutamine. Thus, GS and LGA must be exquisitely regulated to ensure a proper signalling function.

Other implications derived from the current findings are concerned with the glutamatergic regulation in the endocrine pancreas. Recent evidence has indicated that glutamate is a primary signalling molecule that triggers the secondary paracrine-like response of islets [8]. Although glutamate plays an important role in the regulation of hormone secretion, critical issues such as where and how glutamate appears in the islets remain unresolved. Thus, Maechler and Wollheim postulate that glutamate is produced and stored together with insulin in secretory granules of the β-cells [24], while Moriyama and co-workers sustain that glutamate is co-released with glucagon from α-cells in response to low glucose concentration [7]. The idea that glutamate could be produced, stored in and released from either alpha-cells or beta-cells, depending on the stimuli, was apparently reinforced by the finding that both cell types express GA proteins [11]. Often, once the presence of an enzyme has been proved in a tissue or cell, the corresponding enzymatic activity is taken for granted. However, to the best of our knowledge, this is the first time that GA activity has been detected in islet α-cells, suggesting that these cells are capable of producing large amounts of glutamate. In this sense, we are tempted to assign a signalling role to KGA and design the α-cell as the place where the glutamate signal is produced and released from. In contrast, we have failed to prove GA activity in β-cells, despite the fact that these cells abundantly express LGA. Although this negative result does not rule out the so-called glutamate hypothesis, which sustains that this amino acid is produced, co-stored and co-secreted with insulin in β-cells [24], it casts some doubt on the enzyme producing glutamate.

5. Conclusion

In summary, we have developed a new method enabling the detection of GA activity *in situ*. The inorganic phosphate dependence exhibited for this enzymatic activity has been exploited as a strong indicator of specificity. Through the use of this methodology, we have been able to prove, for the first time, the existence of GA activity in α-cells from islets of Langerhans. In contrast, and despite the high levels of LGA protein present in β-cells, we have failed to detect GA activity in these cells. In addition to offering answers to questions previously formulated, the findings reported herein raise new and exciting questions such as, how is LGA regulated in pancreas? Is LGA transiently activated by unknown stimuli? The search for answers to these new questions should help orientate future investigations.

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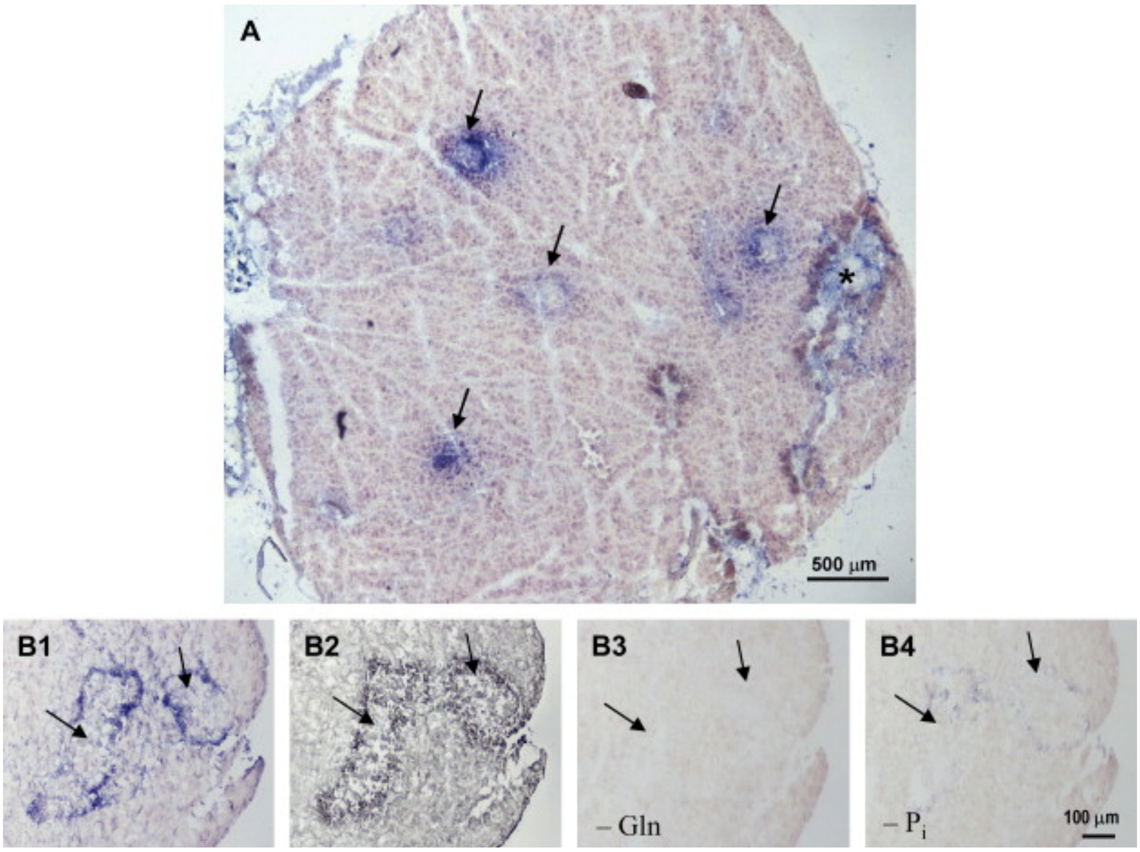
Figures legends

Figure. 1. GA activity is present in the mantle of isles of Langerhans. (A) Panoramic view of a pancreatic section showing GA activity staining. This light micrograph is meant to provide a panoramic, not a detailed, view of the GA staining pattern in unfixed rat pancreas sections. Beside blood vessels (asterisks) and adipose tissue, staining is noted in spheroid structures (arrows) that are scattered throughout the exocrine parenchyma, which appears as a pinkish background. (B) Two different islets (arrows) through four serial sections. B1 corresponds to the GA activity staining which is concentrated into the periphery of the islets. B2 shows how the same islets are immunostained with an anti-glucagon antibody. B3 When glutamine was omitted from the incubation medium no GA activity was observed. B4 proves the dependence on exogenous orthophosphate exhibited for the activity staining.

Figure. 2. Double-labelling with GA activity and endocrine cell markers. Sections were first subjected to GA activity staining (A1, B1 and C1). Afterwards, the same sections were fixed and processed for immunofluorescence using an anti-glucagon antibody as α-cell marker (A2), an anti-insulin antibody as β-cell marker (B2) or an anti-somatostatin antibody as δ-cell marker (C2). The last column (A3, B3 and C3) shows the corresponding overlays. Scale bar 50 μm.

Figure. 3. The *in situ* glutaminase assay is suitable for detection of hepatic LGA. The LGA activity was detected in liver extract (A and B) and liver sections (C and D). The livers were homogenized in 5 ml/g of 300 mM mannitol and 5 mM Hepes, pH 7.4 with a manually operated Potter–Elvehjem glass–Teflon homogenizer. The volume of the homogenate was then doubled by addition of homogenization buffer, followed by centrifugation for 5 min at 1500 × *g* and 4 °C. The supernatant was used as a source of LGA. One millilitre aliquots of the reaction medium either containing or lacking phosphate (see Section 2) were placed into disposable cuvettes and the glutaminase reaction was triggered by adding the indicated volume of crude liver extract. All the incubations were carried out in darkness at 37 °C. (A) The accumulation of formazan (followed at 530 nm) after triggering the reaction with 50 μl of liver extract is shown. (B) In other experiments, different volumes of liver extract were assayed during 5 min. Data represent the mean value of three different experiments after subtracting the absorbance detected in phosphate-omitted samples run in parallel. (C) 16 μm thick cryostat liver sections were subjected to GA activity staining as described in Section 2. As expected for this enzyme in liver, formazan production was heterogeneously distributed through the parenchyma. (D) Importantly, this pattern of staining disappeared when glutamine was omitted from the reaction mixture. Kidney sections processed for GA activity with (E) or without (F) glutamine are shown for comparative purposes. Scale bars 250 μm (C and D) and 500 μm (E and F).



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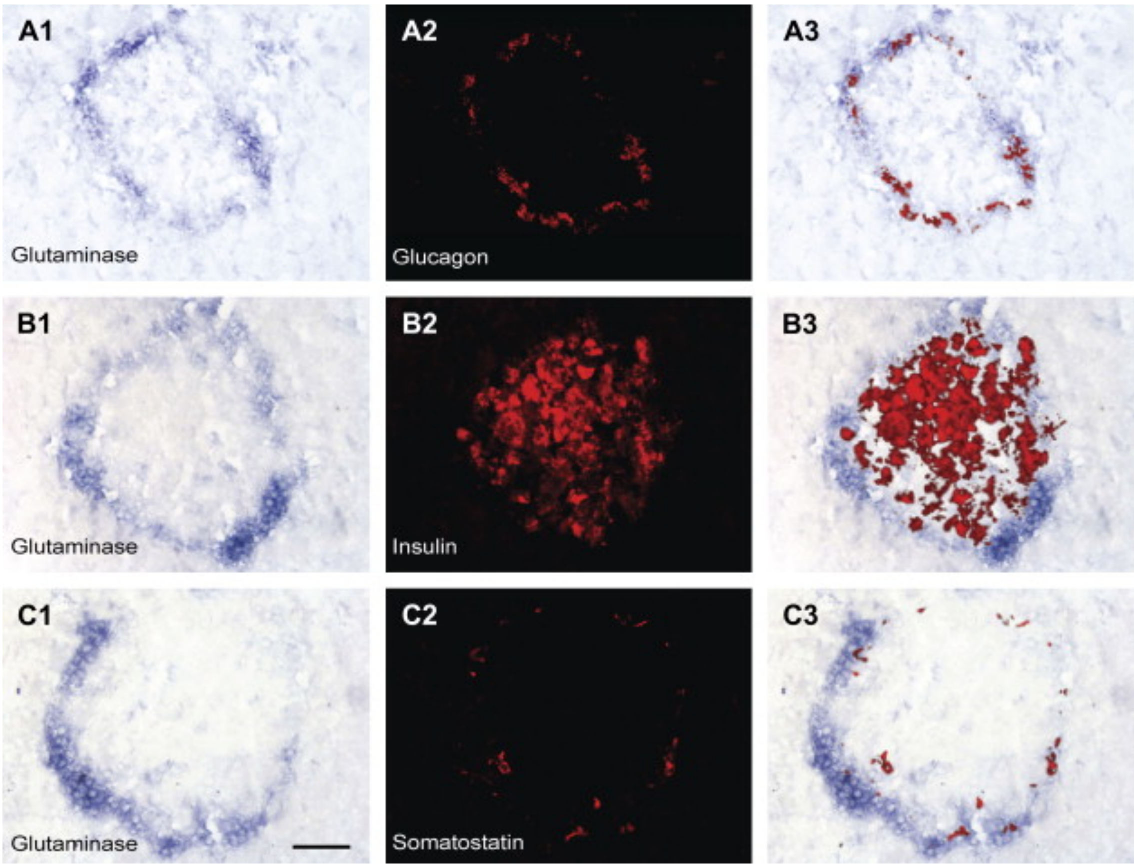
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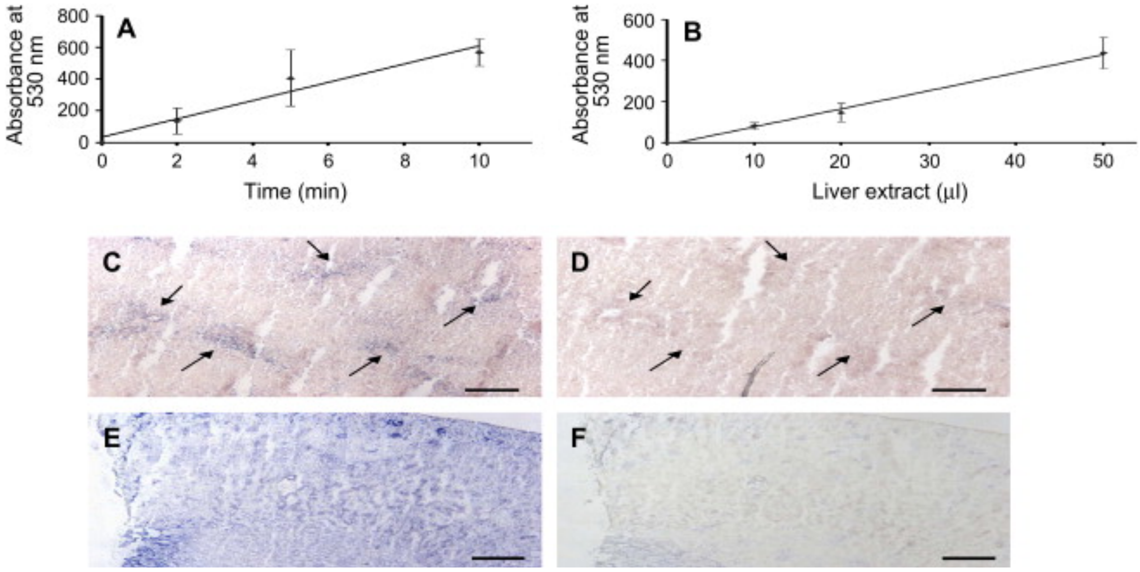
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