

## Research Article

# Insulin-Induced Electrophysiology Changes in Human Pleura Are Mediated via Its Receptor

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**Background.** Insulin directly changes the sheep pleural electrophysiology. The aim of this study was to investigate whether insulin induces similar effects in human pleura, to clarify insulin receptor's involvement, and to demonstrate if glibenclamide (hypoglycemic agent) reverses this effect. **Methods.** Human parietal pleural specimens were mounted in Ussing chambers. Solutions containing insulin or glibenclamide and insulin with anti-insulin antibody, anti-insulin receptor antibody, and glibenclamide were used. The transmesothelial resistance ( $R_{TM}$ ) was determined. Immunohistochemistry for the presence of Insulin Receptors (IRa, IRb) was also performed. **Results.** Insulin increased  $R_{TM}$  within 1st min ( $P = .016$ ), when added mesothelially which was inhibited by the anti-insulin and anti-insulin receptor antibodies. Glibenclamide also eliminated the insulin-induced changes. Immunohistochemistry verified the presence of IRa and IRb. **Conclusion.** Insulin induces electrochemical changes in humans as in sheep via interaction with its receptor. This effect is abolished by glibenclamide.

## 1. Introduction

Insulin is one of the chief mediators of anabolism and glucose controllers. Diabetic patients who receive insulin as treatment may develop a rare but potentially dangerous complication known as “the insulin oedema syndrome” which is characterized from oedema and fluid formation in various sites of the body, ranging from simple ankle oedema to heavy cardiopulmonary manifestations, such as pulmonary oedema, cardiogenic shock and pleural effusions. [1–4].

A possible explanation for the insulin oedema syndrome was proposed to involve the ability of insulin to induce permeability alterations in epithelia such as the distal kidneys, at epithelial cell level, where it induces electrolyte and water retention [5–7] leading to fluid excess in the organism.

However, despite the aforementioned explanation, the formation of pleural effusions in diabetic patients who develop the insulin oedema syndrome remains unexplained. In an effort to explain this event, a direct effect of insulin in pleura was previously demonstrated in sheep [8]; insulin

induced electrochemical changes increasing the pleural trans-mesothelial resistance ( $R_{TM}$ ) by blocking ion transporters such as the amiloride-sensitive  $Na^+$  channels and the ouabain-sensitive  $Na^+/K^+$  pumps who have been implicated in pleural fluid recycling [9]. In that study, the insulin receptors a (IRa) and b (IRb) were demonstrated to be present in sheep pleura, and therefore the aforementioned effect was suggested to be mediated by a possible insulin-insulin receptor interaction [8].

The aim of this study was to investigate if insulin effect on the electrochemical profile of the human parietal pleura follows similar pattern as in sheep, to clarify if an interaction with its receptor is involved, and to additionally investigate if glibenclamide (another hypoglycemic agent also used for diabetes treatment) can reverse this effect.

## 2. Materials and Methods

**2.1. Human Parietal Pleural Specimens.** Intact sheets of human parietal pleura were obtained from forty-four (44)

patients, who underwent thoracic surgery for lung cancer (via thoracotomy or thoracoscopic procedures) for diagnostic and/or therapeutic purposes. The lung mass was not in proximity with the dissected specimens. A piece from each specimen was sent for histopathological examination, and all specimens used in the study were proven to be free of any disease, as per the histopathology report. Patients with pleural effusion prior to the operation, abnormal blood-stream glucose level preoperatively, or history of diabetes were excluded from the study. The remaining specimens were then placed in preoxygenated (bubbled with 95% O<sub>2</sub>—5% CO<sub>2</sub>) Krebs solution, cooled at 4°C, and were transferred to the laboratory within 30 minutes from tissue dissection.

The study was approved by the Local Ethics Committee (Institutional Review Board), and signed consent was obtained from all the participated in the study patients.

**2.2. Electrophysiology Studies.** The KRB solution used throughout the whole study was balanced at pH 7.45 and contained 117.5 mM NaCl, 1.15 mM NaH<sub>2</sub>PO<sub>4</sub>, 24.99 mM NaHCO<sub>3</sub>, 5.65 mM KCl, 1.18 mM MgSO<sub>4</sub>, 2.52 mM CaCl<sub>2</sub> and 5.55 mM Glucose.

The surface of the pleura that faces in vivo the pleural cavity, will be referred to as the *mesothelial surface*, and the surface that faces the chest wall will be referred to as the *interstitial surface*.

The pleural tissues were mounted as planar sheets of tissue in *Ussing-type chambers* [8–10]. The tissue was bathed in Krebs solution on both sides and bubbled continuously with 95% O<sub>2</sub>—5% CO<sub>2</sub> gas mixture, heated to 37°C, in order to ensure tissue viability.

Following the equilibration period and control measurements [8, 10], insulin (bovine pancreas insulin, Sigma Chemical Co., USA) solutions (10<sup>-7</sup> M) were added on the mesothelial and interstitial surface of the specimens ( $n = 7$  experiments for each side) [8]. In other experiments the anti-insulin antibody (Sigma Chemical Co., USA, 50 mg/dl, dilution 1:80000) was added in insulin solutions ( $n = 7$  experiments). Similarly, the anti-insulin receptor antibody (IR, pTyr<sup>972</sup>, Sigma Chemical Co., USA) or the anti-insulin-like growth factor 1 receptor antibody (Sigma Chemical Co., USA) was also added in other insulin solutions ( $n = 7$  experiments). In other experiments glibenclamide (10<sup>-5</sup> M) (Sigma Chemical Co., USA) was added on the mesothelial and interstitial surface of the specimens ( $n = 7$  experiments for each side of the tissue). Finally, insulin 10<sup>-7</sup> M was added in specimens ( $n = 7$ ) pretreated with KRB solution with glibenclamide 10<sup>-5</sup> M for at least 30 minutes prior to the experiments.

PD<sub>TM</sub> was measured 1, 5, 10 and 30 minutes after each solution addition and *Trans-mesothelial Resistance* ( $R_{TM}$ ) was calculated from PD<sub>TM</sub> [8–10].

**2.3. Statistical Analysis.** Statistical analysis was performed using the statistical package SPSS ver. 10.00 for Windows (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., USA). Data are expressed as Mean  $R_{TM}$  ( $\Omega \cdot \text{cm}^2$ )  $\pm$  Standard Error of Mean (S.E.). Statistical significance

between pairs was determined by student's paired *t*-test whereas between multiple groups by ANOVA (Bonferoni's post hoc). *P* values less than .05 were considered significant.

**2.4. Immunohistochemistry.** Tissue sections (3  $\mu$ ) from parietal human pleura were dried onto slides overnight at 60°. After deparaffinization in xylene and rehydration in decreasing ethanol solutions, slides were heated in target retrieval solution (pH 9, DAKO Denmark) for 25 minutes in a microwave oven (LG WAVEDOM 850 Watt). The sections were cooled, washed in PBS, and incubated in 0.3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. After washing with PBS, 75  $\mu$ L of blocking buffer (DAKO, Carpinteria CA, USA) was added to each section for 1 hour. Then, the sections were incubated with the primary antibody to Insulin receptor a (rabbit polyclonal Santa Cruz Biotechnology, Santa Cruz CA) and Insulin receptor b, (rabbit polyclonal Santa Cruz Biotechnology, Santa Cruz CA) at 1:300 and 1:200 dilution, respectively, for 1 hour in room temperature. After the incubation, envision fluid (polymer-peroxidase method, EnVision+ /HRP, DAKO Denmark) was added, followed by incubation for 30 minutes. The slides were counterstained with hematoxylin and mounted. Tissue sections from pancreas and liver were used as positive controls. Blood vessels wall in the mesothelium specimens were considered also as positive controls. For the negative control the incubation step with the primary antibody was omitted.

### 3. Results

**3.1. Effect of Insulin in Human Parietal Pleura.** Addition of insulin on the mesothelial surface increased  $R_{TM}$  rapidly, within the 1st minute (from  $20.99 \pm 0.5 \Omega \cdot \text{cm}^2$  to  $22.85 \pm 0.6 \Omega \cdot \text{cm}^2$ ,  $dR_{TM}$   $1.86 \Omega \cdot \text{cm}^2$ , versus control  $P = .016$ ). This effect lasted for 5 min ( $22.11 \pm 0.6 \Omega \cdot \text{cm}^2$ , versus control,  $P = .026$ ), and  $R_{TM}$  was decreased thereafter till baseline ( $20.92 \pm 0.6 \Omega \cdot \text{cm}^2$ , versus control,  $P > .05$ ) after 30 minutes (Figure 1(a)). Little effect was observed interstitially ( $20.99 \pm 0.5 \Omega \cdot \text{cm}^2$  to  $21.47 \pm 0.6 \Omega \cdot \text{cm}^2$ ,  $dR_{TM}$   $0.48 \Omega \cdot \text{cm}^2$ , versus control  $P > .05$ ) (Figure 1(b)).

**3.2. Effect of Anti-Insulin Antibody on Insulin-Induced Alterations.** The anti-insulin antibody totally inhibited the insulin-induced effect (from  $22.85 \pm 0.6 \Omega \cdot \text{cm}^2$  to  $21.05 \pm 0.6 \Omega \cdot \text{cm}^2$   $P = .01$ , versus control  $P > .05$ , Figure 2).

**3.3. Effect of Anti-Insulin Receptor Antibody on Insulin-Induced Alterations.** The anti-insulin receptor antagonist also totally inhibited insulin-induced effect (from  $22.85 \pm 0.6 \Omega \cdot \text{cm}^2$  to  $20.95 \pm 0.6 \Omega \cdot \text{cm}^2$   $P = .01$ , versus control  $P > .05$ , Figure 3).

**3.4. Effect of Anti-Insulin-Like Growth Factor 1 (IGF) Receptor Antibody on Insulin-Induced Alterations.** The anti-IGF-1 receptor antagonist did not inhibit the insulin-induced effect (from  $22.85 \pm 0.6 \Omega \cdot \text{cm}^2$  to  $22.54 \pm 0.5 \Omega \cdot \text{cm}^2$   $P > .05$ , versus control  $P = .014$ , Figure 3).

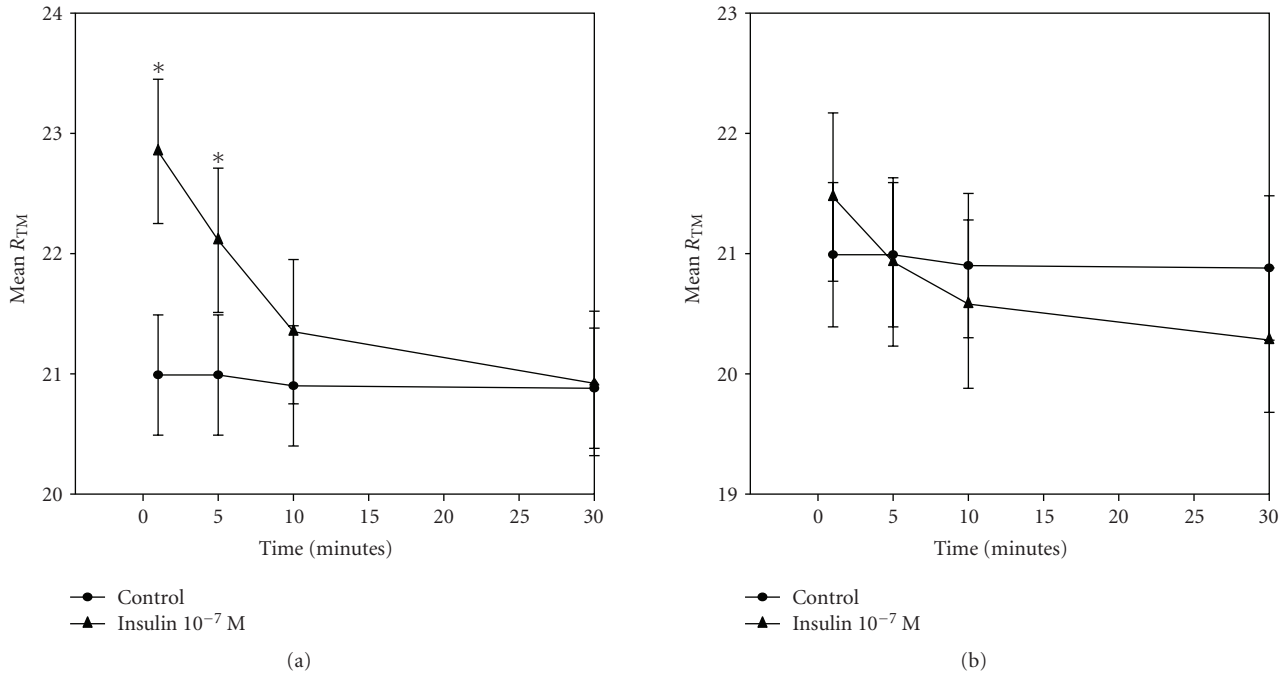


FIGURE 1: Effect of insulin  $10^{-7}$  M addition on the Trans-mesothelial Resistance ( $R_{TM}$ ) when added on the mesothelial (a) and interstitial (b) surface of human parietal pleura, by time. Values are expressed as Mean Trans-mesothelial Resistance ( $\Omega \cdot \text{cm}^2$ )  $\pm$  Standard Error of Mean;  $n = 7$  experiments. \* $P < .05$  versus control.

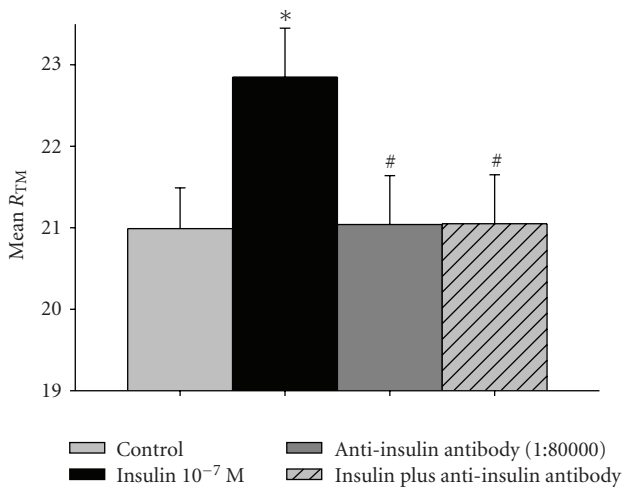


FIGURE 2: Effect of Anti-Insulin antibody on the insulin-induced electrochemical alterations in human parietal pleura. Values are expressed as Mean of Trans-mesothelial Resistance ( $\Omega \cdot \text{cm}^2$ )  $\pm$  Standard Error of Mean of  $n = 7$  for each set of experiments. \* $P < .05$  versus control, # $P < .05$  versus insulin.

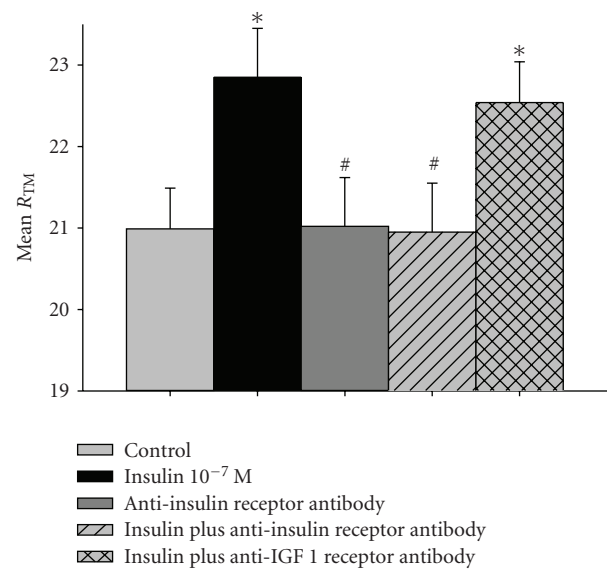


FIGURE 3: Effect of Anti-Insulin Receptor antibody and Anti-IGF 1 Receptor antibody on the insulin-induced electrochemical alterations in human parietal pleura. Values are expressed as Mean of Trans-mesothelial Resistance ( $\Omega \cdot \text{cm}^2$ )  $\pm$  Standard Error of Mean of  $n = 7$  for each set of experiments. \* $P < .05$  versus control, # $P < .05$  versus insulin.

**3.5. Effect of Glibenclamide in Human Parietal Pleura and on Insulin-Induced Alterations.** Addition of glibenclamide had no effect on  $R_{TM}$  either when added mesothelially (weak increase of  $0.54 \Omega \cdot \text{cm}^2$ , versus control  $P > .05$ , Figure 4) or interstitially.  $R_{TM}$  remained near baseline throughout the experiments. Glibenclamide abolished the insulin-induced

(Figure 4)  $R_{TM}$  increase from the 1st min of coaddition (from  $20.99 \pm 0.6 \Omega \cdot \text{cm}^2$  to  $21.30 \pm 0.5 \Omega \cdot \text{cm}^2$ ,  $dR_{TM} 0.31 \Omega \cdot \text{cm}^2$ , versus insulin  $P = .022$ , versus control  $P > .05$ ).

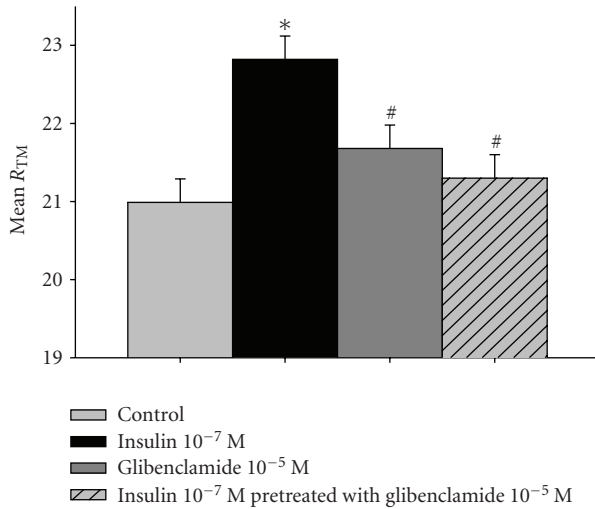


FIGURE 4: Effect of Glibenclamide  $10^{-5}$  M on the insulin-induced electrochemical alterations in human parietal pleura. Values are expressed as Mean of Trans-mesothelial Resistance ( $\Omega \cdot \text{cm}^2$ )  $\pm$  Standard Error of Mean of  $n = 7$  for each set of experiments. \* $P < .05$  versus control, # $P < .05$  versus insulin.

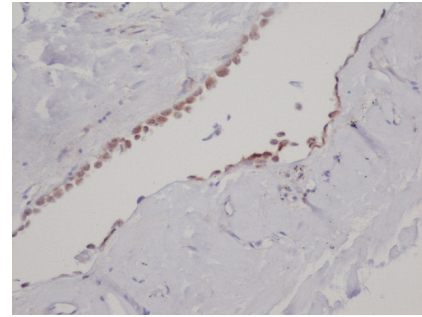
**3.6. Detection of IRa and IRb in Human Parietal Pleura.** Mesothelial cells showed positive immunostaining for IRa and IRb. The immunoreactivity was cytoplasmic (Figures 5(a) and 5(b)). The distribution of immunoreactivity was diffuse. Staining intensity was even and convincing.

## 4. Discussion

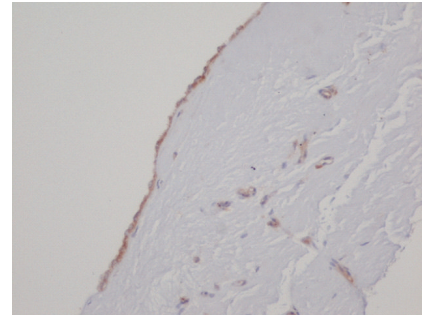
The main finding of this study is that insulin induced electrochemical changes in human parietal pleura when added on the mesothelial surface. This effect is elicited after interaction of insulin with its receptors which were identified as per the immunohistochemistry to be present in human parietal pleura. This insulin-induced effect is reversed by another commonly used hypoglycaemic agent, glibenclamide, which had a weak effect on the electrochemical profile of the human parietal pleura.

Insulin induces similar effects in other tissues such as the toad urinary bladder where insulin increased short circuit current within the first 5 minutes towards the mesothelial side of tissue [11]. Insulin showed an increase of  $PD_{TM}$  of alveolar type II cells when added mesothelially [12, 13]. Interstitial effect of insulin due to diffusion was cited in toad urinary bladder [11]. Weak interstitial effect was observed in this study possibly due to diffusion [14, 15] or because of remnants of fat tissue or blood clots [16]. In kidney cells insulin is known to stimulate amiloride-sensitive  $\text{Na}^+$  channels when added apically [6, 7, 11].

In the present study, insulin was used in a concentration of  $10^{-7}$  M, given that this concentration was previously shown to be the least effective concentration in sheep [17]. In insulin-treated humans, insulin levels rarely reach such high concentrations (i.e., in poorly controlled patients), suggesting that the pleura can be directly stimulated by



(a)



(b)

FIGURE 5: (a) IRa cytoplasmic immunoreactivity in mesothelial cells of parietal mesothelial tissue (immunoperoxidase stain, original magnification  $2 \times 20$ ). (b) IRb positivity in mesothelial cells of parietal mesothelial tissue (immunoperoxidase stain, original magnification  $2 \times 20$ ).

insulin only with high concentrations, explaining in this way the rarity of pleural effusions during insulin therapy [2]. Insulin is possibly diffused into the pleural cavity according to bloodstream levels following diabetes treatment. This high concentration could also explain the fact that in pleura insulin decreases the permeability rather than augments it, as is the case in kidneys. High insulin concentrations can produce different effect patterns in different epithelia or even in the epithelia of the same target organs that is, the kidneys were even interstitial effect was recorded when insulin concentrations used were high [18].

It has been commonly demonstrated that insulin augments glucose uptake via interaction with its receptor. Additionally, insulin interferes with  $\text{Na}^+$  transportation in epithelial tissues via interaction with its receptor [6, 7, 19, 20]. Similar permeability regulation by insulin via a receptor-mediated process was shown in T84 colonic cells [21]. Insulin receptors were demonstrated to be more abundant on the basolateral side of human bronchial epithelial cells [22]. Results from the present study show that insulin and its interaction with its receptors a (IRa) and b (IRb) induce the observed electrochemical alterations in the human parietal pleura.

From all the aforementioned, insulin induced a comparable electrochemical effect in sheep and human pleura. Additionally, the insulin receptors a (IRa) and b (IRb) were

also shown to exist in both species. Therefore, electrophysiological and histopathological observations present many similarities in sheep and human pleura suggesting that sheep can be an acceptable animal model for observations that may be extrapolated to humans. This finding is important given that the human tissue is hard to be obtained due to the fact that consent is needed the healthy subjects are not subjected to surgery whereas its stripping leads to bleeding [8].

IGF-1 also produces transcellular ion fluxes across epithelial tissues and has been implicated to change the permeability that is, of the kidneys [23, 24] after interaction with its receptor. IGF-1 and insulin receptors have similar structure, and insulin may interact with IGF-1 receptor. If insulin induces its effect by binding to the IGF-1 receptor, then the inhibition of the insulin receptor would not have inhibited the insulin's effect in pleura, given that the IGF-1 receptor would have been free to bind with insulin. If insulin induces its effect by binding to the insulin/IGF-1 receptor, then the inhibition of the insulin receptor would have partially inhibited the insulin's effect in pleura. The results of the coaddition of insulin with the anti-IGF-1 receptor antibody in some specimens suggest that the IGF-1 receptor is not involved with the insulin effect in pleura at this at least concentration ( $10^{-7}$  M). However, more research is warranted in order to clarify the involvement of the IGF-1 receptor in the insulin effect in pleura.

Glibenclamide is an antidiabetic agent which enhances insulin production by  $\beta$ -pancreatic cells via inhibition of adenosine triphosphate sensitive  $K^+$  channels (K(ATP)) [25, 26]. Such channels are present in kidneys [27, 28] constituting glibenclamide as an agent interfering with the electrolyte transportation [29–31]. However, its involvement in the function of mitochondrial K(ATP) attributed to this drug a possible harmful effect [32]. Such harmful effect in terms of electrophysiology was not supported by our results. More importantly, glibenclamide inhibited the insulin-induced electrochemical effect in pleura. This inhibition is explainable as it depletes the cellular ATP content, and therefore the insulin-insulin receptor binding may be hindered. The blockage of  $K^+$  transportation via K(ATP) channels by glibenclamide needs further clarification in pleura since its addition did not induce electrochemical and permeability changes suggesting that such channels do not exist in pleura. Apart from its hypoglycemic role, glibenclamide was additionally chosen for its effects in kidneys and myocardium [33, 34].

Results from the present study indicate that theoretically insulin presence in the pleural cavity makes the pleural membrane less permeable [8, 35], and this event may provide an additional explanation for the formation of pleural effusion in insulin-treated diabetic patients. Insulin may therefore present oedematous properties by altering the electrophysiological profile and consequently interfere with pleural recycling [35]. This oedematous effect can be reversed by another hypoglycaemic agent such as glibenclamide. This observation, although at experimental level, may provide an alternative treatment option for clinicians who encounter this rare complication of pleural effusion formation during insulin therapy.

## 5. Conclusions

In conclusion, insulin induced electrophysiology alterations of the human parietal pleura which were similar with the effect produced in sheep pleura and is mediated by interacting with its receptor. This finding alongside with the fact that these receptors were demonstrated to be present in human parietal pleura as in sheep implicates the similarity of the parietal pleura of the two species. The hypoglycaemic agent glibenclamide totally inhibited this effect without inducing intense electrophysiological alterations of the human parietal pleura.

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