Genistein: effect on the ion transport of the intestine of seawater adapted, *Anguilla anguilla*

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Abstract. Genistein is a phytoestrogen belonging to the class of isoflavones. Like other phytoestrogens it is considered as an ambient contaminant that can reach the aquatic environment by sewage treatment plant and pulp mill effluents or wasterwater in which they are present as pharmaceutical compounds. These substances can have deleterious effects on the reproductive system of fish, due to their structural similarity with estrogen. The investigations of the effects of pollutants on the European eel (*Anguilla anguilla*) is of great interest because this species can bioccumulate large amounts of lipophilic contaminants that can reach toxic concentrations when the fish utilize the stored fat, thus contributing to the observed decline of eel stocks. In this study we investigated the effects of the phytoestrogen genistein on the ion transport of the intestine of *A. anguilla* adapted to sea-water, since it was shown that this substance can affect different membrane ion transporters. For this purpose we tested the effect of the isoflavone on the transepithelial parameters (I_{sc}= short circuit current and g_t= transepithelial conductance) of the isolated intestine by employing Ussing chamber experiments. We showed that genistein reduced I_{sc} at all the concentrations tested (from 10^{-6} M to 10^{-4} M) with a maximal effect observed with 10^{-4} M genistein. Experiments performed with known inhibitors of ion transporters suggested that the cotransport Na/K/2Cl is the target of the isoflavone. In view of the important role played by this transport mechanism in the adaptation of the animal to seawater, we conclude that the phytoestrogen can have deleterious effects on this fish species, not only by interfering with the endocrine system, but also by impairing osmoregulation.

Keywords: *Anguilliformes*, Eel intestine, ion transport, genistein.

INTRODUCTION

Genistein is a phytoestrogen, a plant compound structurally similar to animal estrogens (Wang et al.,1996). Phytoestrogens belong to three different classes: lignans, isoflavones and coumestans, genistein is an isoflavone (Dixon, 2004). Phytoestrogens have the role to defend the plant against herbivores and fungal pathogens and contribute to flower coloration (Wynne-Edwards, 2001). Due to their structural similarity with estrogen they can bind to estrogen receptors and act as weak estrogens or weak antiestrogens. It was proposed that they have evolved as strategy to protect the plant from herbivores by interfering with the reproductive ability of these animals (Hughes, 1998).

There is an increasing interest for these substances in human medicine for the treatment of menopausal and postmenopausal symptoms (Cheng et al., 2004; Beck et al., 2005; Mc Carty, 2006) and of cardiovascular disease (Setchell & Cassidy, 1999; Altavilla et al., 2004) and for cancer protection (Adlercreutz, 1995; Wu et al., 2002; Hilakivi-Clarke et al., 2006).

However they are also considered as environmental contaminants that can negatively affect the reproductive system of both terrestrial and aquatic animals, for this reason they are termed "endocrine distruptors" (Crisp et al., 1998). Terrestrial animals can be exposed to phytoestrogen contained in the food while aquatic animals can be exposed to phytoestrogen of various sources. They can reach the aquatic environment by effluents of sewage treatment plants (Stumpf et al., 1996; Spengler et al., 2001; Pawloski et al., 2003, 2004; Puglisi et al., 2003) by pulp mill effluents (Cook et al., 1996; Kiparissis et al., 2001; Mahmood-Khan & Hall, 2003) and by wasterwater in which they could be present as pharmaceutical compounds (Boxall, 2004).

The contamination of the aquatic environment by estrogenic substances has attracted the attention of many researchers for their effects on the reproductive system (Bortone et al., 1989; Jobling et al., 1998; Sumpter, 1998; Tyler & Routledge, 1998) and behaviour (Coltfelter & Rodriquez, 2006) of fish. However the phytoestrogen genistein could also produce detrimental effects on fish osmoregulation since it was shown that it alter ion transport in different epithelia (Martinez et al., 1998; Gimenez et al., 1998; Andersson et al., 2003). For this reason it is interesting to test the effect of the soy isoflavone genistein on ion transport of the marine teleost intestine since this organ has an important osmoregulatory role in the adaptation to seawater.

The studies of the effect of pollution on the eel, *A. anguilla*, are very important because the stocks of this fish are in decline and there is a body of evidence that anthropogenic factors as pollution have contributed to the decline of this species (see Geeraerts & Belpaire, 2010), observed since the mid -1960s (Dekker, 2002). Due to their lifestyle eels are able to bioaccumulate contaminants, especially lipophilic, during their feeding stage to a level making them not suitable for the human consuption (Harrad & Smith, 1999; Bilau et al., 2007). Differently from the iteroparous fish, the eels do not have contaminant loss associated with the reproductive cycles. As long as the contaminant are stored their effects are minimal, but when the eels start the migration to seawater, they utilize the stored fat and the contaminants can be released and reach toxic plasma level (see Geeraerts & Belpaire, 2010).

In view of the above considerations we decide to study the effect of the lipophilic substance genistein on ion transport of the isolated intestine of *Anguilla anguilla* adapted to seawater.

MATERIALS AND METHODS

The middle intestine of seawater-acclimated *A. anguilla* was removed, stripped of longitudinal and circular layers using two pairs of fine forceps and mounted vertically in a modified Ussing chamber (CHM6, World Precision Instruments, Berlin, Germany; membrane area: 0.13 cm^2), where it was perfused on both sides by isotonic teleost Ringer solution (NaCl 133 mM, KCl 3.2 mM, $NaHCO_3$ 20 mM, $MgCl_2$ 1.4 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 0.8 mM, glucose 20 mM; osmolarity: 315 mOsm kg^{-1}). The temperature of the perfusing Ringer solution was kept constant at 18°C.

- Tissues were connected to an automatic short-circuit current device (DVC-1000, World Precision Instruments) by four Ag/AgCl electrodes (two voltage electrodes and two current electrodes) that made contact with the bathing solutions via agar-Ringer filled cartridges.
- I_{sc} was measured by passage of sufficient current through Ag/AgCl electrodes to reduce

the spontaneous V_t to zero (resistance of the chamber fluid was subtracted automatically). The preparations were kept open circuited throughout the experiments, except for a few seconds every 5 min for recording the short-circuit current (I_{sc}). The negative sign of I_{sc} indicates current flowing from the apical to the basolateral side; g_t was calculated according to Ohm's law (g_t =I/V). The transepithelial parameters were allowed to stabilize (about 30 min) before starting any experimental procedure.

All the drugs used were obtained from SIGMA (St Louis, MO). They were prepared as stock solutions in the appropriate solvent according to manufacturer's instruction and diluted in the experimental chamber in order to obtain the desired concentration. Genistein, glybenclamide, NPPB [5-nitro-2(3-phenylpropylamino) benzoic acid)] and bumetanide were dissolved in DMSO (dimethyl sulfoxide) to prepare concentrated solutions which were diluted in the experimental solutions. The end concentration of DMSO was 0.1%; preliminary experiments showed that it did not alter the control electrophysiological parameters. Genistein was added to both the serosal and the mucosal side, glybenclamide, NPPB and bumetanide to the luminal side. Except for genistein, for which dose-response experiments were performed, the drug concentrations used were determined from earlier our studies.

All data are expressed as mean ± SE. Data were normally distributed (Kolmogorov-Smirnov test, $p < 0.05$). In each set of experiments, each tissue served as its own control. Statistical analyses were performed using GraphPad Prism 5. The effect of the different treatments on control I_{sc} was evaluated by one way ANOVA. Dunnett's multiple comparison test was applied for post hoc comparison. Differences were considered significant at $p < 0.05$.

RESULTS

We performed dose-response experiments in order to know the concentration useful to produce the maximal inhibitory effect on the short circuit current. For this purpose, when the transepitelial parameters of the isolated intestine had reached stable values in the control conditions, we added progressively larger amounts of genistein to both mucosal and serosal bath. Each addition was followed by a restabilization period during which I_{sc} reached a new steady state; thereafter the next higher concentration of the drug was tested. As showed in Fig. 1, the lowest concentration used (10^{-6} M) reduced I_{sc} by 40%, a further 20% reduction was observed with 10^{-5} M while I_{sc} was almost nullified by 10^{-4} M genistein. Higher concentrations were without effect (not shown).

In Fig. 2 the time course of the effect on I_{sc} of the maximal concentration of the isoflavone is reported in a typical experiment representative of 4. The mean values of 5 experiments are reported in Tab. 1. The drug reduced I_{sc} but was not able to modify the transepithelial conductance (Tab. 1).

In order to verify whether the genistein target was a luminal Cl^- channel, the isoflavone was tested in tissues preincubated from the luminal side with either NPPB (10^{-4} M) or glybenclamide (10^{-4} M), two different Cl^- channel blockers. Fig. 3, in which a typical experiment is reported, shows that the addition of glybenclamide produced negligible effects on the control I_{sc} while the following addition of genistein nullified the transepithelial parameter. The mean values of four experiments are reported in Tab. 1.

Genistein produced similar effects in tissues preincubated with luminal NPPB (Fig. 4).

Fig. 5 reports a typical experiment in which genistein was tested after bumetanide (10^{-5}

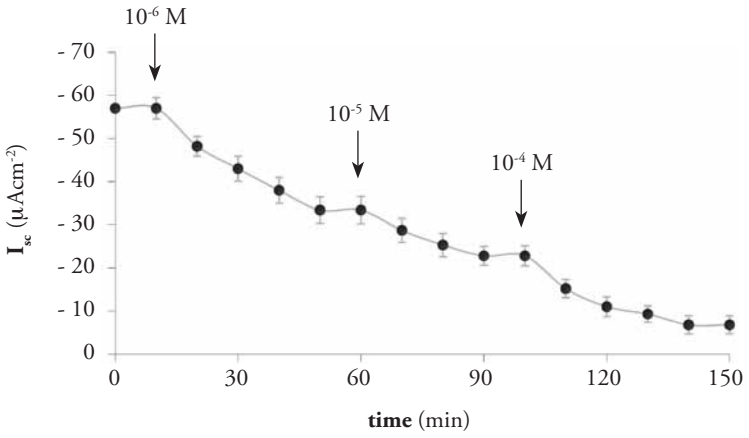

Fig. 1 – Time course of the effect on I_{sc} of various concentrations of genistein. n (number of tissues) = 4.

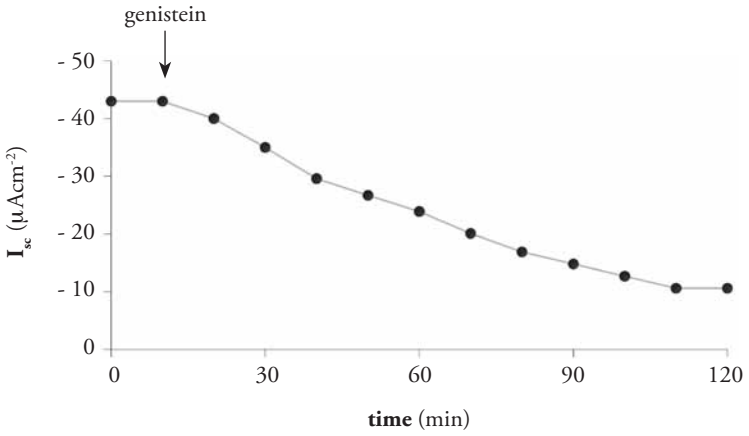

Fig. 2 – Time course of the effect of bilateral genistein (10^{-4} M) on I_{sc} in a typical experiment representative of 5.

M) produced its maximal inhibitory effect on I_{sc}, it is evident that genistein produced a small decrease of I_{sc}; this decrease was not statistically significant (Tab. 1).

CONCLUSION

This study shows that the phytoestrogen genistein reduces I_{sc} in a dose-dependent manner, its effect is already evident at micromolar concentrations (Fig. 1). These concentrations could be realistic since eels accumulate high concentrations of lipophilic xenobiotics in the adipose tissue. The uptake of xenobiotics takes place by gills skin and contaminated food (see Geeraerts & Belpaire, 2010 for a review). It is known that eels are resistant and can live in the sediment polluted by different contaminant. They live for a mean of 5.9 years for males and 8.7 years for females in fresh water (Vollestad, 1992) where they can bioaccumulate xenobi-

Tab. 1 – Effect of genistein (10^{-4} M) on I_{sc} and tissue conductance (g_t) in control tissues and in tissue preincubated with bumetanide (10^{-5} M) or glybenclamide (10^{-4} M). Values are means ± ES; n= number of experiments. The - sign of I_{sc} indicates a current flow from mucosal to serosal side *p < 0.001 vs corresponding control.

	I_{sc} ($\mu A cm^{-2}$)	g_t ($mScm^{-2}$)	n
Control	-47 ± 5.9	40.8 ± 4.5	5
genistein	-8.8 ± 2.2	40.5 ± 3.9	
Control	-49 ± 4.9	40.8 ± 4.5	4
glybenclamide	-48 ± 5.1	40.2 ± 4.1	
+genistein	-6.7 ± 4.2*	40.5 ± 3.9	
Control	-56.1 ± 5.1	40.8 ± 4.5	4
bumetanide	-7.9 ± 0.8*	40.7 ± 4.2	
+genistein	-6.5 ± 0.7	40.5 ± 3.9	

otics reaching a maximum level prior to start the migration to the sea (Tapie et al., 2011; sea also Geeraerts & Belpaire, 2010 for a review).

The observation that genistein reduces I_{sc}, a measure of transepithelial ion transport across a variety of epithelial membranes including intestinal mucosa (Gonzalez Bosc et al., 2001; Evans et al., 2004; Karaki & Kuwahara, 2004), suggests that the drug is able to modulate the ion movement across the epithelium.

The possibility that genistein acts on the paracellular route is ruled out by the observation that the isoflavonol does not alter the transepithelial conductance (Tab. 1) that in a leaky epithelium, like the intestine is, depends on the conductance of the tight junctions (Powel, 1982). It is conceivable that the targets of the phytoestrogen are the mechanisms responsible of the transcellular ion movement. The reduction of I_{sc}, due to the net Cl^- absorption across eel

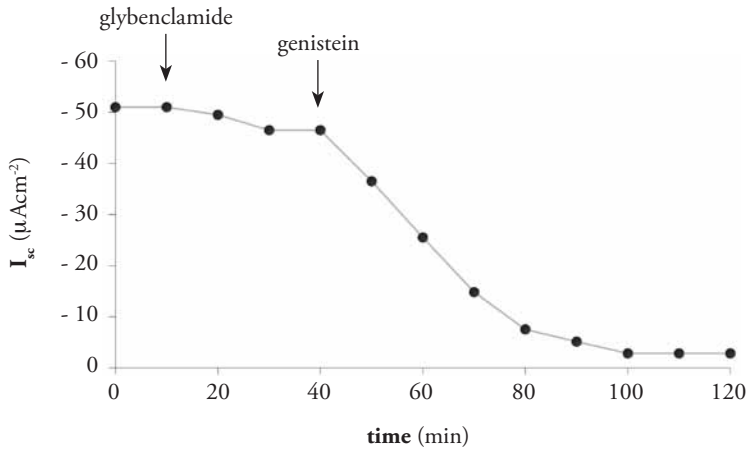

Fig. 3 – Effect of the bilateral addition of 10^{-4} M genistein after than glybenclamide (10^{-4} M) had produced its maximal effect on I_{sc}. A typical experiment representative of 4.

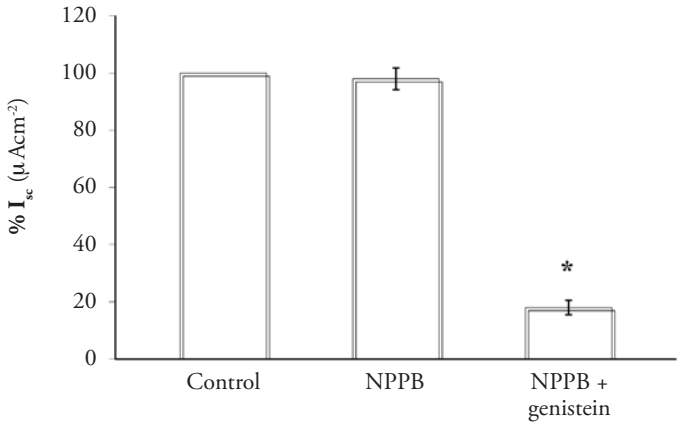

Fig. 4 – Effect on I_{sc} of the bilateral addition of genistein (10^{-4} M) in tissues preincubated with luminal NPPB (10^{-4} M). The values are means ± SE of 4 experiments for each condition. *p < 0.001 vs corresponding control.

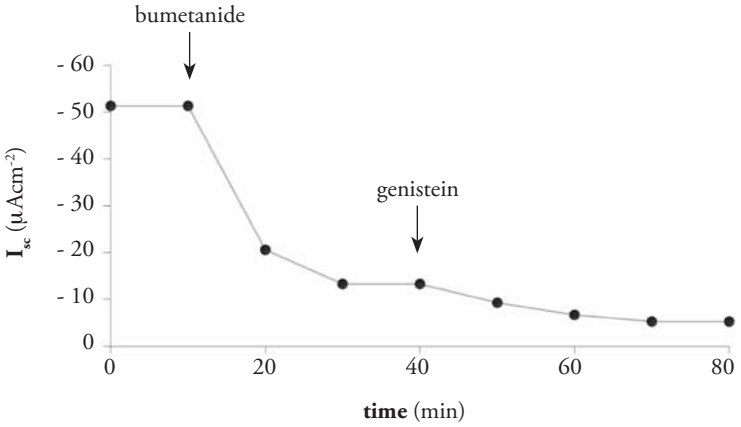

Fig. 5 – Effect of the bilateral addition of 10^{-4} M genistein after than bumetanide (10^{-5} M) had produced its maximal effect on I_{sc}. A typical experiment representative of 4.

intestine (Trischitta et al., 1992a; b) let us to formulate two hypothesis: a) genistein stimulates Cl^- secretion; b) genistein reduces Cl^- absorption.

The first hypothesis is supported by the observation that the opening of Cl^- channels is involved in the vasorelaxant action of genistein in the isolated rat aorta (Valero et al., 2006). To verify the first hypothesis we tested the drug in the presence of luminal glybenclamide, known as inhibitor of the CFTR, Cistis Fibrosis Conductance Regulator (Schultz et al., 1999). The presence of luminal channels sensitive to glibenclamide has been already shown in the eel intestine, their opening was modulated by the flavonol quercetin (Trischitta & Faggio, 2006).

On the contrary it is unlikely that the soy isoflavonoid genistein stimulates Cl^- secretion because in tissues preincubated in the presence of the glybenclamide, genistein elicited its usu-

al effect on I_{sc} (Fig. 3). The experiments performed in the presence of 10^{-4} M NPPB, another drug known to inhibit CFTR in many epithelia (Schultz et al., 1999), strengthened our conclusion (Fig. 4).

The most probable hypothesis to explain the reduction of I_{sc} in the presence of genistein is that the phytoestrogen inhibits Cl^- absorption by acting on the luminal Na-K-2Cl. It is known that this electroneutral symport is the main transport mechanism located at the brush border membrane of the enterocyte of many teleosts adapted to seawater (Field et al.,1978; Musch et al., 1982; Halm et al., 1985a; b; Trischitta et al, 2004), including *A. anguilla* (Trischitta et al., 1992a). This transporter, responsible of the Cl^- entry into the cell, is sensitive to loop diuretic, the most effective being bumetanide (Trischitta et al., 1992b). The hypothesis that the target of genistein is the cotransport Na-K-2Cl arises from the observation that the isoflavonol was ineffective when tested in tissues in which bumetanide had elicited its maximal inhibitory effect on I_{sc} (Fig. 5 and Tab. 1). Indeed an inhibitory effect of genistein on this ion transport mechanism has been already demonstrated in the rat Henle loop (Martinez et al., 1998).

The ability of genistein to inhibit the Na-K-2Cl cotransport could have detrimental effects on the eel during its migration to seawater for the considerations reported below. As previously stated eel can accumulate large amounts of contaminant, especially lipophilic, during its life in freshwater. These contaminants are released during migration towards the reproductive sites in the Sargasso Sea when the fish utilize the stored fat.

Our previous studies showed that the absorption of Cl^- is mediated by the Na-K-2Cl both in freshwater and in seawater eels, differently from other teleosts in which the adaptation to different salinities change the basic mechanism for salt absorption (Lau, 1985; Zuidema et al., 1985; Agenaou et al., 1989). We suggested that the adaptation to seawater of the eels involves an increased activity of the symporter. Based on these observations it could be argued that the inhibition of the Na-K-2Cl by genistein affects the osmoregulatory function of the eel intestine. This suggests that the drug can have deleterious effects not only by interesting with the endocrine system but also by impairing the ability of the eels to adapt to seawater.

RIASSUNTO

La genisteina è un fitoestrogeno appartenente alla classe degli isoflavoni. Come altri fitoestrogeni è considerato un contaminante ambientale sia per gli animali terrestri che per quelli acquatici. I primi possono essere esposti ai fitoestrogeni contenuti nei cibi, i secondi a quelli che giungono nell'ambiente acquatico mediante diverse fonti: i liquami del trattamento delle piante, gli effluenti delle cartiere e, infine, le acque di scarico industriali o domestiche, nelle quali possono essere presenti come composti farmaceutici. E' noto che i fitoestrogeni, compresa la genisteina, vengono oggi largamente impiegati nel controllo dei disturbi della menopausa e della postmenopausa e dei disturbi cardiovascolari nonché nella protezione dal cancro. Uno smaltimento non corretto di tali farmaci, come peraltro di tutti farmaci, è responsabile del loro accumulo nell'ambiente acquatico. Esistono molte prove che i fitoestrogeni hanno effetti deleteri sugli organismi acquatici a causa della loro somiglianza strutturale con gli estrogeni e sono pertanto indicati come "distruttori endocrini". È stato suggerito che l'inquinamento ambientale può avere gravi effetti sull'anguilla europea, *Anguilla anguilla*, ed essere probabilmente una delle cause del depauperamento di tale specie, osservato sin dalla

metà degli anni '60. Questi pesci infatti, per il loro particolare ciclo vitale, bioaccumulano elevate quantità di xenobiotici, specialmente di natura lipofila, durante la vita in acqua dolce. Fintantoché sono immagazzinate, tali sostanze producono effetti minimi. Durante le migrazioni verso il mare le anguille utilizzano i grassi di deposito e pertanto gli xenobiotici accumulati vengono rilasciati massivamente e possono raggiungere livelli tossici. Di conseguenza, lo studio dell'impatto degli inquinanti su tali teleostei è di grande importanza. In questo lavoro abbiamo investigato l'effetto del fitoestrogeno genisteina sul trasporto di sali dell'intestino di *Anguilla anguilla* adattata all'acqua di mare, poiché studi recenti suggeriscono che questa sostanza influenza l'attività di molti trasporti ionici di membrana. A tal fine è stato testato l'effetto dell'isoflavone sui parametri elettrici transepiteliali (I_{sc}= corrente di corto circuito e g_t conduttanza transepiteliale) dell'intestino dell'anguilla isolato e montato nella cameretta di Ussing, utilizzando la tecnica della cortocircuitazione. I dati ottenuti hanno dimostrato che la genisteina riduceva I_{sc} (espressione dell'assobimento netto di Cl^-), a tutte le concentrazioni testate (da 10^{-6} M a 10^{-4} M), con un effetto massimale alla concentrazione di 10^{-4} M. Esperimenti condotti in presenza di inibitori specifici di differenti trasportatori di membrana hanno suggerito che il principale bersaglio dell' isoflavone è il cotrasporto Na/K/2Cl, bumetanide sensibile. Visto l'importante ruolo giocato da questo trasportatore nell'adattamento dell'anguilla all'acqua di mare, è possibile concludere che il fitoestrogeno può avere effetti deleteri su questo pesce, non solo perchè interferisce con il sistema endocrino, ma anche perchè compromette i meccanismi di osmoregolazione.

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