Original article

The strong functional association between energy metabolism and reproduction: a major driver for sex-specific physiopathology

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Summary. In the entire animal kingdom, there are strong interrelations between the metabolic and reproductive systems. These mechanisms have been preserved throughout evolution, because meant to limit reproduction in a nutritionally unfavorable environment. In oviparous animals, the liver exerts its control of fertility by producing the proteins essential for the maturation of the egg only in the presence of gonadal (estrogens) and nutritional (proteins) stimuli. In mammals, hepatic control of the successful progression of the ovulatory cycle is maintained; however, the augmented demands of the reproductive system associated with the growth of the placenta, embryogenesis and lactation have increased significantly the mechanisms controlling the mutual interactions between liver metabolic activity and reproductive functions. The hypothesis that we put forward here is that an evolutionary pressure was exerted on the liver of female mammals to perfect such complex mechanisms, determining a major divergence of the hepatic metabolism in females and males. The hepatic isoform of the estrogen receptor (ERα) has a major role in the development and maintenance of male and female sexual dimorphism in liver functions. The identification of the major involvement of liver ER in the preservation of female energy metabolism points to this receptor as a potential target for a future, efficacious post-menopause hormone replacement therapy.

Key words: liver, sexual dimorphism, estrogens, energy metabolism.

La stretta associazione funzionale tra metabolismo energetico e riproduzione: un driver importante per la fisiopatologia sessuale specifica

Riassunto. Tradizionalmente si è sempre ritenuto che, con l'eccezione delle attività strettamente associate alla riproduzione, non esistessero grandi differenze fisiologiche tra i mammiferi dei due sessi e che alcune diversità nell'incidenza di malattie fossero strettamente legate al comportamento sociale più che alla presenza del cromosoma maschile nel corredo genetico. Il crescente numero di studi che identifica maggiori differenze di sesso nell'incidenza di patologie molto diverse tra loro e nella risposta ai trattamenti farmacologici apre la strada a una revisione critica del nostro modo di affrontare la sperimentazione in campo biomedico, ponendo più enfasi negli studi comparativi nei due sessi e nella comprensione della reale origine e significato biologico di tali differenze. Questo articolo vuole presentare una chiave di lettura per tali differenze che nasce dalla nostra competenza nello studio dei recettori degli estrogeni. Studiando il meccanismo d'azione degli estrogeni ci siamo resi conto che, insieme alle gonadi, il fegato è il maggiore bersaglio d questi ormoni che hanno un enorme controllo su tutto il metabolismo energetico femminile. Tale controllo è reso necessario per adattare il metabolismo alle necessità riproduttive che nella femmina di mammifero sono cresciute enormemente rispetto agli ovipari. Quindi l'ipotesi che noi perseguiamo è che nei più di cento milioni di anni dalla comparsa dei mammiferi sulla terra, le nuove responsabilità riproduttive dei mammiferi di sesso femminile abbiano indotto una selezione di quegli individui che più erano in grado di adattare il proprio metabolismo alle funzioni riproduttive. Tale tipo di selezione ha fatto divergere in modo molto profondo le capacità metaboliche dei due sessi con enormi conseguenze fisiopatologiche anche esacerbate da un ambiente in cui la disponibilità di cibo e la sua qualità sono enormemente cambiate rispetto a quelle che di fatto hanno plasmato l'evoluzione della specie.

Parole chiave: fegato, dimorfismo sessuale, estrogeni, metabolismo energetico.

Introduction

Traditionally, the concept of sexual differentiation has been associated with the organs that are directly involved in reproduction and genetic sex has been considered to have little or no influence on the physiopathology of non-reproductive organs. This view is now challenged by the increasing number of reports demonstrating that sex has a significant impact on the incidence of a large number of pathologies that, apparently, are barely involved with reproduction; among these, we can mention metabolic diseases (diabetes)[1], lung pathologies (pulmonary arterial hypertension)[2], neurological and neuropsychiatric disorders (Parkinson, schizophrenia, depression, migraine)[3], bone and joint pathologies (arthritis)[4] and immune dysfunctions (autoimmune diseases)[5,6]. In addition, pharmaco-epidemiological surveys have demonstrated that the number of side effects of drugs is significantly higher in females[7,8]

further indicating that the therapies we are adopting do not have the same effects in the two sexes, possibly because most preclinical and clinical studies are carried out in males.

These lines of evidence have stimulated research in the field of gender/sex medicine and pharmacology and we are now witnessing an increase in the number of reports demonstrating how relevant is the physiological divide between the two sexes is in the animal kingdom. Most of these studies define the existence of pathophysiological differences associated with genetic sex, but the mere description of such differences does not help to understand their biological roots and does not provide a framework of thought that enables us to predict potential harmful effects of drugs or set up appropriate prevention of pathologies. The aim of this article is to reason on the potential origins of sex differences without overlooking those associated with gender effects, (meaning the differences associated with cultural habits that in humans may represent a risk factor for a number of pathologies).

Sex steroids: how large is their influence on the onset and maintenance of sex differences?

In embryogenesis, sex organs originate from a common primordium and early on in gestation the transcriptional activation of the *SRY* gene (located in the Y chromosome) leads to the synthesis of the testis determining factor, a protein that is able to deviate the fate of ovarian cell progenitors toward the generation of the male gonads, the testis. Once developed, the tissues forming male and female gonads (testis and ovaries) acquire all the enzymatic equipment necessary to respond to pituitary hormones and synthesize the sex steroid hormones that will drive the development of internal and external reproductive organs present in the newborns. At puberty, the gonads initiate the synthesis of the sex steroids that will accomplish the molding of the male/female phenotype inducing the development of secondary sexual characteristics such as the female breast, the pattern of pubic and facial hair, the deepening of the voice, etc. All this is well known, but less understood is the fact that organs other than those classically associated with the male/female phenotype undergo a sex-differentiation process: for instance, the neuronal circuits that regulate the synthesis of several pituitary hormones are sexually differentiated. This sexual differentiation occurs much before puberty, at the end of embryo formation (or first few days of neonatal life, depending on the mammalian species), and is triggered by the surge of testosterone synthesis occurring perinatally in male testis. Testosterone, which is converted into estradiol by brain aromatase, "de-feminizes" brain circuits relevant for the regulation of sexual behavior and other autonomic functions thus organizing the neural circuits that are able to respond to sex hormones at puberty and in adults. In the absence of testosterone, these brain functions maintain their female-like features. A clear demonstration of this phenomenon can be obtained by treating neonatally genetic female mice with testosterone or estradiol; once these females reach adulthood, they will display behaviors clearly associated with the male phenotype: first of all, the mounting behavior and the lack of male acceptance of males[9].

For a long time it was believed that this "masculinization" paradigm was restricted to neuronal circuits strictly associated with sexual and parental behavior. However, two studies recently published in the literature showed that this "defeminization/masculinization" process may occur in cells other than neurons. In the first study, it was demonstrated that following neonatal treatment with sex hormones male microglia acquire a more activated phenotype than females and this characteristic is maintained throughout adult life[10]. The second study provided evidence that the same is true for the liver, an organ that appears to undergo sexual differentiation at birth with the testosterone surge[11].

Therefore, two sex steroids (mainly estradiol and perhaps testosterone) play a major role in development by preparing and organizing cells and tissues that during adult life will be their targets and where they will directly regulate gene expression by activating their receptors, *bona fide,* hormone-regulated transcription factors, estrogen receptors alpha and beta and the androgen receptor[12].

Estrogen receptors are present in most mammalian cells, including hepatic cells

Estrogens may regulate the activity of their target cells by binding to two type of receptors: the two intracellular isoforms, estrogen receptors alpha (ERα) and beta (ERβ) and the transmembrane G-protein coupled receptor GPER[12]. ERα and ERβ are transcription factors maintained inactive by specific inhibitory proteins; once bound by the cognate or phosphorylated ligand, ERα and ERβ release the inhibitory proteins and become able to recognize and associate with specific DNA sequences (so-called estrogen responsive elements, or ERE). Here, the ERs interact with the co-regulators and the transcription machinery thus participating in the control of the state of transcription of specific genes. Alternatively, these intracellular receptors may interfere with the signaling of membrane receptors or transcription factors by direct protein-protein interactions. Intracellular ERs may undergo a series of post-translational modifications such as phosphorylation, acetylation

and others[13]; in case of palmitoylation, intracellular ERs may be integrated in the cell membrane where they can still signal intracellularly after binding estrogens. G-protein coupled estrogen receptors (GPER) are membrane receptors that bind estrogens with high affinity and initiate rapid, estrogen-receptor signaling mediated by the G proteins[12].

In mammals, ERs are present in most cell types thus pointing to a widespread action of these hormones. The study of ER biological functions has been carried out primarily in cell systems and little was known with regard to their involvement and significance in the physiology of the entire body. To shed light in the ER physiology, several years ago, we conceived a mouse reporter aimed at revealing *spatio*-temporally the state of transcriptional activity of intracellular ERs. This transgenic mouse was conceived to enable the ubiquitous expression of the firefly luciferase gene under the control of a multimerized synthetic ERE[14]. In this mouse, the expression of luciferase was shown to be strictly associated with ER transcriptional activation. Using this reporter system, we discovered that, together with the gonads, the liver is the organ with the most intense ER transcriptional activity that may be ascribed mainly to ERα, the ER isoform most expressed in the liver. Investigations into the ER transcriptional activity in living, fertile, female mice showed that in the liver, contrary to the other non-reproductive organs, ER transcriptional activity is synchronous with all reproductive organs thus pointing to a potential functional association between hepatic and reproductive functions[14]. Intriguingly, the transcriptional activity of the hepatic ERα was induced by estrogens, but also by amino acids. This observation was extremely puzzling until we realized that the hepatic ER activity is indispensable for the synthesis of the proteins essential for egg maturation. Indeed, in all oviparous animals, the liver and the ovaries, together, are the key players in a feedback-feedforward cycle where the gonads synthesize the estrogens that reach the liver and activate ERs to synthesize the proteins indispensable for the progression of the fertile cycle. All this is possible only when a sufficient pool of amino acids is present in the hepatic tissue. The requirement of amino acids guarantees the continuation of the reproductive cycle only in conditions of satiety: in case of prolonged starvation the lack of essential dietary amino acids stops the fertile cycle. This mechanism has been a safeguard for the extinction caused by an uncontrolled proliferation in an environment poor of nutrients where famine might compromise the life of the parents together with their offspring. Understandably, this biological defense mechanism is very well preserved in all animal species where the liver or its ancestor is the gauge of the level of nutrition below which procreation has to be arrested[15].

Liver and reproductive functions in mammals

Our past work has demonstrated that in mice, like oviparous animals, a protein-poor diet results in decreased synthesis of IGF-1 in the liver, thus bringing the reproductive cycle to a halt[15]. This is not surprising considering the essential role for the preservation of the species of the control of energy metabolism on reproduction, but, unlike oviparous animals, in mammals the reproductive functions do not end with ovulation and more requests for energetic support arise from the placenta, the growing embryo and the organs responsible for the lactation of the newborn. This must have tremendously increased the complexity of the metabolic and reproductive interplay. In fact, while in oviparous animals, the production of the proteins necessary for the maturation of the egg is a task carried out by the liver, in female mammals the metabolic systems must be able to adapt to the each stage of reproduction characterized by a different level and type of energy request.

We demonstrated that throughout the reproductive cycle the liver changes its metabolism[11, 16]; it is plausible, therefore, that during gestation and even more so during lactation the liver employs specific strategies to meet the diversified energy requirements characterizing each reproductive status. Therefore, specific mechanisms should be in place to allow the liver and possibly the other metabolic organs to adapt their metabolism to guarantee a successful embryogenesis and growth of offspring. Supporting this view, experimental evidence in animals and humans has shown that liver metabolism is highly affected by ovulation, pregnancy and lactation. During the early phases of gestation, the female liver maximizes its lipogenic potential and uses all available substrates to synthesize storage lipids: this is necessary to ensure that the growth of the embryo can continue also in case of periods of famine[17], later on, the liver adapts its metabolic pathways to spare the amino acids and glucose needed in the last stages of embryo development. The liver is therefore responsible for the switch from anabolic to catabolic metabolism reported in the mid-end gestation of most mammalian species. With lactation, the liver engages in the massive production of glucose enabling the mammary glands to create milk sugars and the glycerol backbone for milk fat.

These mechanisms are the result of about 120 million years of evolutionary selective pressure initiated with the appearance of mammals on earth. However, such pressure must have been exerted only in females because male reproductive strategies in mammals are the same as in oviparous species. This differential evolutionary history must have significantly differentiated the liver in a sex-dependent way. Indeed, we know that in mammals the liver is the organ showing the highest degree of sexual dimorphism. Several studies have dem-

onstrated that the hepatic P450 enzymes are expressed differently in the sexes: the enzyme CYP3A4 is much less expressed in males. Since CYP3A4 is responsible for the catabolism of benzodiazepines, tricyclic antidepressants and others, this finding by itself may explain the differential effects that drugs have in the two sexes[17]. Other sex differences were reported for the catabolism of endogenous hormones (such as cortisol and testosterone) and for metabolic functions. With regard to metabolic activities, fertile females have much higher rates of liver fatty acid uptake and esterification, higher VLDL-TG synthesis and secretion and more efficient cholesterol utilization than males[18]. The transcriptome reflects these sex-specific hepatic functions and most recently the combination of transcriptomics and metabolomics data have made it possible to demonstrate, at least in mice, the major strategic differences between the two sexes when dealing with short-term food deprivation: at the first sign of food shortage, the male liver stops the production of molecules for energy storage, while the female liver adapts its enzymatic apparatus to use all available substrates (including amino acids) to continue triglyceride accumulation. This observation was done with females at metestrus, a phase of the cycle in which the ovarian follicles initiate their growth, i.e., a phase in which females prepare for a potential pregnancy by storing energy to support the rapid growth of the placenta and the initial phases of embryo development. This strategy, besides increasing the stored energy, may also be aimed at maintaining a low content of amino acids in the liver to improve its ability to become rapidly aware of decreased food ingestion[11].

The hepatic estrogen receptor plays a major role in female liver metabolism: this was demonstrated in a series of biochemical and metabolic studies carried out in conditional knock-out mice with the selective ablation of hepatic ERα[11,16], thus suggesting that this receptor is functionally very important for sex-specific dimorphism on liver metabolism. Prior studies ascribed the sex-specific hepatic metabolism to the pituitary growth hormone (GH) that shows a sexually dimorphic pattern of pulsatile secretion and is an important determinant of growth, liver enzyme function and insulin-like growth factor I (IGF-I) expression. In males, GH is secreted at 3-4 intervals and results in major plasma changes of the hormone; in females, the pulses are less frequent and of lower intensity, thus GH plasma concentration does not change as much as in males. GH secretion by the pituitary gland is regulated by the nutritional status (fasting, exercise or low glucose levels reduce GH) and by estrogens. We do not know how the sex-specific secretion of ERα regulates liver functions: we cannot rule out that GH by inducing IGF-1 synthesis may regulate the hepatic ERα activity, through unliganded activation[19].

Conclusions: understanding sex-specific physiology and precision medicine

In female mice, the virtuous circle perfected to maintain all nutrients in excess in safe deposits and to use and reuse cholesterol for reproductive purposes may explain why women, during their fertile age, are less susceptible than males to metabolic diseases and the related cardiovascular consequences. However, this subtle balance is under the control of estrogens, thus at the cessation of ovarian functions the entire system becomes dysfunctional: there is a rapid accumulation of fat in the liver, increased incidence of pathologies such as non-alcoholic fatty liver disease, metabolic syndrome and related cardiovascular incidences. The fat deposited in the liver initiates inflammatory reactions triggering a generalized state of inflammation that synergizes and precipitates the incidence of most of the diseases associated with aging that are characterized by a strong inflammatory component: arteriosclerosis, neurological disorders, immune diseases. This may explain why in females the ageing process accelerates after menopause. In fact, female metabolic activities shaped over millions of years of evolution in an environment poor of nutrients are now confronted with the abundant availability of obesogenic compounds which precipitates the effect of menopause as indicated by studies on the prevalence of obesity in women after the age of 50 and the relative increase in their health costs[20].

Our current limited knowledge of the differential mechanism governing male and female physiology limits our comprehension of the etiology of a number of pathologies and our ability to establish appropriate prevention and therapeutic interventions. We hope that the large amount of correlative studies carried out in the two sexes are soon converted into mechanistic hypothesis. We strongly believe that putting sex difference studies in the perspective of evolution highlights the value of preclinical studies for the understanding of the molecular bases of sex differences. The expansion of such studies will facilitate the progress of the field enabling the exploitation of the molecular tools so far available and of limited application in clinical practice, such as genetic manipulations, but also the full control of experimental factors such as environment, diet and exercise.

Finally, having established the relevance of estrogens female metabolism, it would be most important to identify novel and more efficacious means for a replacement therapy aimed at maintaining the protective effects of these hormones[21]. We hope for an increased attention to the necessities of women particularly in the many years after their fertile life to identify therapies that can diminish their personal and societal costs associated with aging.

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Key messages
■ Energy metabolism and reproductive functions are mutually regulated in the entire animal kingdom.
■ In female mammals, the liver is highly involved in sustaining reproductive functions.
■ In mammals, the changes in reproductive strategies has determined a major functional divergence of hepatic metabolic strategies in males and females.
■ The cessation of ovarian functions determines a major change in energy metabolism that may precipitate the progression of numerous diseases associated with aging.
■ The liver estrogen receptor may represent a novel, relevant target for post-menopause hormone replacement therapies.

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