Voltage-dependent Potassium Channels Kv1.3 and Kv1.5 in Human Fetus

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Key Words

Potassium channels • Human fetus • Development • Nerve • Muscle • Hematopoietic

Abstract

Voltage-dependent K⁺ channels (Kv) control repolarization and membrane potential in electrically excitable cells. In addition, Kv channels are involved in the maintenance of vascular smooth muscle tone, insulin release, epithelial K⁺ transport, cell proliferation and leukocyte activation. Kv1.3 and Kv1.5 are widely distributed throughout the body and are involved in a variety of physiological processes taking place in the immune system, brain and muscle. Since the developmental pattern of Kv channels has an essential role in the maturing human, we aimed to study Kv1.3 and Kv1.5 channels in 8-10 week human fetal tissues. We chose that gestational age because all organs are in place and the nervous system, although not fully developed. However, the human embryo is undergoing major changes, which will lead to a defined adult pattern. Our results indicated that numerous tissues expressed Kv1.3 and Kv1.5. While Kv1.3 overlapped with the central and peripheral nervous systems, Kv1.5 was mostly localized in the

central nervous system. In addition, both channels were abundantly expressed in the hematopoietic fetal liver. Finally, Kv1.5 heavily stained skeletal muscle and heart, whereas Kv1.3 was slightly present. This is the first study to analyze Kv1.3 and Kv1.5 in human during the beginning of fetal development.

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Introduction

Voltage-dependent K⁺ (Kv) channels play a critical role in a wide variety of physiological processes, including cardiac action potentials, muscle contraction, neuronal excitability, insulin secretion, epithelial K⁺ transport, and cell volume regulation [1]. In addition, some Kv channels are also involved in activation, proliferation and differentiation of mammalian cells [2].

Kv channels have a putative topology of six membrane-spanning domains and the core channel structure is a homo- or heterotetramer, which may be associated with several regulatory subunits [1]. The Kv superfamily comprises twelve subfamilies (Kv1-Kv12) [3]. Kv1.3 and Kv1.5 are members of the Shaker (Kv1) family implicated in tissue differentiation and cell growth

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[2]. Kv1.3 was first cloned in brain, but its expression is more widespread than previously thought [4, 5]. Kv1.3 controls leukocyte membrane potential and function during immune system responses [6]. In addition, Kv1.3 is also expressed in olfactory bulb, hippocampus, epithelia, adipose tissue and skeletal muscle [7-10]. Kv1.5 was isolated from human ventricle, but it has also been shown to be expressed in the atria [11]. Kv1.5 is also present in kidney, the immune system, and in the brain to a lesser extent [5, 12-14]. Both proteins, which share expression in the immune system, form heterotetrameric channels [14-16]. These oligomeric forms generate multiple biophysically and pharmacologically distinct channels [14-16]. The level of expression of both subunits influences the degree of proliferation, differentiation or activation of some cell types [16, 17]. The physiological importance of Kv1.3 and Kv1.5 has been demonstrated by genetic variations that trigger diseases. For example, Kv1.3 inherited polymorphisms lead to insulin resistance and an impaired glucose metabolism, while mutations of Kv1.5 trigger atrial fibrillations [18, 19].

Kv channels show differential expression during development [20-25]. However, most data refer to adult tissues. Nevertheless, important differences in Kv channel expression patterns during neonatal development have been documented [20-25]. Thus, a precisely defined expression pattern for these channels during development will contribute to a better understanding of Kv channels in adult phenotypes and physiological functions. Kv1.3 and Kv1.5 channels follow a distinct developmental expression profile, which eventually defines a clear adult phenotype and influences final physiological function [22, 23]. For instance, impaired expression of Kv1.3 in T-effector memory cells is involved in the onset of juvenile multiple sclerosis [26]. In addition, a Kv1.3 deficiency alters insulin sensitivity and glucose tolerance [18]. Therefore, the Kv1.3 channel represents a candidate gene for type 2 diabetes [9, 18]. On the other hand, Kv1.5 is markedly decreased by hypothyroidism and deletion of the Kv1.5-partner *kcne2*, which impairs channel expression, triggers cardiopathies with a marked form of hypothyroidism with deleterious results in neonates [27-29].

Nothing is known about the expression of Kv1.3 and Kv1.5 channels during the early stages of human development. Therefore, we analyze Kv1.3 and Kv1.5 protein expression in the 8-10 week human fetus. While every organ is in position at this age, many of the processes of cell maturation still occur. The brain undergoes profound changes during this period, which

include cell proliferation, cell differentiation and neuronal migration. Although the nervous system is defined at this age, maturation of higher functions of brain development occurs much later in pregnancy [30]. Finally, the fetal liver serves as the initial hematopoietic tissue and some skeletal muscle myogenic markers are expressed [31, 32]. We found that Kv1.3 was present in most epithelia and the liver. Kv1.3 was mostly coexpressed with S-100 protein, which mostly marks nerve structures, and it was also detected in muscle. On the other hand, Kv1.5 was highly expressed in heart, liver and muscle. Interestingly, although Kv1.5 was abundantly found in the central nervous system, it stained no peripheral nerves.

Materials and Methods

Patient tissue characteristics and sample processing

Three apparently healthy human fetuses (8-10 weeks gestational age) were obtained from the Department of Pathology of Vall d'Hebron University Hospital (Barcelona, Spain). Causes of death were spontaneous abortion (amnion infection) or induced legal terminations. The developmental age of the fetuses was determined by the date of the last menstrual bleeding. Weight and length measurements evaluated at the autopsy were used to assure proper gestational development.

Samples were fixed in neutral formalin and embedded in paraffin for histopathological study. Tissue examination was performed by pathologists with light microscopy examination using conventional hematoxylin and eosin staining and an ancillary immunohistochemical staining for S100 when necessary. We used S-100, which heavily stains nerve structures, because is mostly present in cells derived from the neural crest (Schwann cells, melanocytes, and glial cells). However, this protein is also present in chondrocytes, adipocytes, myoepithelial cells, macrophages, Langerhans cells, dendritic cells, and keratinocytes. Representative images are shown.

Antibodies and immunohistochemistry

Immunohistochemical staining using the avidin-biotin-peroxidase technique was performed for each antibody as previously described [5]. Samples were fixed in formalin, embedded in paraffin, and received routine histopathological examination. Paraffin embedded tissue blocks were cut into 4 μ m sections and mounted onto poly-L-lysine-coated glass slides. Sections were deparaffined in xylene and rehydrated in graded alcohol. For antigen retrieval, slides were heated either in a pressure cooker in 10 mM citric acid monohydrate, pH 6.0, for 5 min (Kv1.5) or in a 98°C water bath in 10 mM citric acid monohydrate, pH 9.0, for 40 min (Kv1.3). Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxidase blocking solution for 10 min. Samples were immunoblotted with anti-Kv1.3 (1:70) and anti-Kv1.5

Fig. 1. Kv1.3 and Kv1.5 specific staining was abolished by the presence of an antigen peptide and by omitting anti-Kv1.3 and anti Kv1.5 primary antibodies. A-C, Kv1.3. A, Kv1.3 polyclonal primary antibody (1:70) was used on a lymphoma sample as described in Materials and methods. B, Kv1.3 primary antibody was incubated in the presence of Kv1.3 antigen peptide (Alomone) following manufacturer's instructions. C, Lymph node specimen was prepared and processed as described in materials and methods but the anti-Kv1.3 primary antibody was omitted in the incubation. D-F, Kv1.5. D, Kv1.5 polyclonal primary antibody (1:150) was used on a myometrial smooth muscle sample as described in Materials and methods. E, Kv1.5 primary antibody was incubated in the presence of Kv1.5 antigen peptide (Alomone) following manufacturer's instructions. F, Myometrial smooth muscle specimen was prepared and processed as described in materials and methods but the anti-Kv1.5 primary antibody was omitted in the incubation.

(1:100) polyclonal antibodies (Alomone) for 1 h, followed by incubation with a HRP-labeled polymer anti-rabbit (DakoCytomation) for 30 min. When necessary, we used a rabbit polyclonal primary antibody against S-100 protein for immunohistochemical staining of nervous tissue (1:500, DakoCytomation). Immunohistochemical analysis was performed with the EnVision system (DakoCytomation). All samples were counterstained with hematoxylin, dehydrated and mounted. Negative controls were performed in all cases by omitting the primary antibody and in the presence of the antigen peptide (Alomone) following manufacturer's instructions (Fig. 1).

Results

Kv1.3 and Kv1.5 in fetal head tissues

Figure 2 shows two different axial planes of a 10 week human fetal head. While Fig. 2 A and C show representative brain pictures including brain ventricles and thalamus, Fig. 2 B and D illustrate detailed slices including retinal epithelium, nasal fossa respiratory epithelium, trigeminal ganglion and pituitary gland (hypophysis). Kv1.3 was homogeneously distributed throughout the tissues (Fig. 2 A and B). However, Kv1.5 staining was more

Fig. 2. Immunohistochemistry of Kv1.3 and Kv1.5 in the 10 week human fetal head. Two representative serial axial planes are shown. Six independent pictures captured at 20X are combined to depict the whole head. A and C, superior section. B and D, inferior section. A and B, Kv1.3. C and D, Kv1.5. Labels in A and C: GM, germinal matrix; C, primitive cortex; T, thalamus; LV, lateral ventricle; EL, ependymal layer. Labels in B and D: L, lens; R, retina; NF, nasal fossa; OM, orbital muscle; TG, trigeminal ganglia; H, hypophysis infundibula (pituitary gland); M, mesencephalon. Boxes indicate specific regions further analyzed in Fig. 3.

selective (Fig. 2 C and D).

When regions, highlighted with boxes in panel B and D, were analyzed in detail in Figure 3, selective staining was observed. Kv1.3 was present at relatively low levels in retina, trigeminal ganglia and meninges (dura mater), hypophysis, nasal fossa epithelia and orbital muscle (Fig. 3 A, C, E, G and I, respectively), whereas Kv1.5 staining was expressed much stronger in all of these tissues (Fig. 3 B, D, F, H and J). It is noteworthy that Kv1.5 was highly expressed along the lining surrounding immunonegative tissues. In addition to the retinal pigmented epithelium, which was intrinsically stained, Kv1.3 and Kv1.5 stained the rest of the retinal layers.

Kv1.3 and Kv1.5 channel expression in the body

Figure 4 represents consecutive slices from an 8 week human fetus. Unlike brain and head tissues, Kv1.3 stained more selectively throughout the body (Fig. 4 A). Although some tissues are analyzed in depth below, it is

Fig. 3. Detailed analysis of Kv1.3 and Kv1.5 expression in several parts from the 10 week human fetal head. Sections (100X) of boxes highlighted in Fig. 2 were analyzed for Kv1.3 and Kv1.5. A and B, eye; C and D, trigeminal ganglia; E and F, hypophysis; G and H, nasal fossa; I and J, orbital muscle. PE, retinal pigmented epithelium; R, retina; DM, dura mater (meninges); OM, orbital muscle.

noteworthy that spinal cord (SC) and intercostals nerves (NE) were heavily stained. Peripheral nerves were also notably stained (see below). In addition, Kv1.3 was present at low levels in cardiac (H) and several skeletal muscles, such as diaphragm (DI). Strong Kv1.3 signals were observed in several epithelia. Thus, small intestine (SI), colon (CO), and several epithelial layers in kidney (K), lung (LU) and pancreatic ducts (PA) were positively stained. Unlike Kv1.3, Kv1.5 differentially stained the nervous system (Fig. 4 B). While dorsal ganglia (DRG) and spinal cord were Kv1.5 immuno-positive, the peripheral nervous system was negative. Intense Kv1.5 signal was also observed in epithelia, such as skin (SK), and it was also expressed in several muscles. It is important to point out that stronger signals for both Kv1.3 and Kv1.5 were found in the adrenal gland (AG), pancreas (PA) and the liver (LI).

To further identify tissues that expressed Kv1.3 and Kv1.5 during fetal development in humans, we analyzed several families of organs. Figure 5 shows the expression of both channels in electrically excitable tissues, such as

Fig. 4. Immunohistochemistry of Kv1.3 and Kv1.5 in the 8 week human fetal body. Two representative serialized planes are shown. Nine independent pictures captured at 20X are combined to depict the whole body. A, Kv1.3. B, Kv1.5. Boxes indicate specific regions analyzed in further figures. Labels in A: VB, vertebral body; NE, intercostal nerves; H, heart; AG, adrenal gland; SI, small intestine; DI, diaphragm; UL, upper limb (hand); LL, lower limb (leg). Labels in B: SC, spinal cord; DRG, dorsal root ganglia; LU, lungs; K, kidney; CO, colon; LI, liver; PA, pancreas; OV, ovary; AR, upper limb (arm); PL, chorionic villi (placenta); SK, skin; RI, ribs.

Fig. 5. Kv1.3 and Kv1.5 expression in nerve and heart. A serialized group of slices were analyzed for the Kv1.3, Kv1.5 and S-100 (neural marker) expression. A-C, Dorsal root ganglia (40X); D-F, peripheral nerve (40X); G and H, heart (200X); V, ventricle; A, atria Arrows in panels G and H indicate nerve (N).

Fig. 6. Kv1.3 and Kv1.5 expression in upper and lower limbs. A serial group of slices were analyzed for the Kv1.3, Kv1.5 and S-100 (neural marker) expression. A and B, Upper limb (hand) (40X); C-E, upper limb (arm) (40X); F and H, lower limb (leg) (100X). N, nerves; E, elbow.

nerve and heart. The expression analysis of dorsal root ganglia revealed that both Kv1.3 (Fig. 5 A) and Kv1.5 (Fig. 5 B) overlapped with the S-100 protein (Fig. 5 C). However, when peripheral nerves were analyzed (Fig. 5 D-F), only Kv1.3 stained significantly these tissues (Fig. 5 D). Kv1.5 was cloned from human heart where it conducts the I_{kur} current [11, 33]; accordingly, cardiac tissue was heavily stained. However, we found no major differences in immuno-signal between atria and ventricles (Fig. 5 H). Unlike Kv1.5, the role of Kv1.3 in cardiac tissue is uncertain, although the presence of Kv1.3 has been documented in muscle [9]. In this context, Kv1.3 uniformly stained fetal heart (Fig. 5 G).

We next analyzed some skeletal muscles and related regions within the upper and lower limbs. Figure 6 represents a serial analysis in hand, arm and leg of Kv1.3 (Fig. 6 A, C and F), Kv1.5 (Fig. 6 B, D and G) and the S-100 protein (Fig. 6 E and H). While Kv1.3 slightly stained skeletal muscle, there was a stronger signal in peripheral nerves. It is interesting to note that Kv1.3 and S-100 strongly colocalized in the arm and leg. In contrast, Kv1.5 notably stained skeletal muscles and was almost absent in nerves.

Data in Figure 4 indicate that Kv1.3 and Kv1.5 were present in epithelia. Therefore, we further analyzed several organs that share epithelial characteristics. Figure 7 depicts the expression of Kv1.3 and Kv1.5 in small intestine (SI), kidney (K), liver (LI), lung (LU) and skin (SK). Both proteins were notably present in SI epithelia (E). However, while Kv1.5 strongly stained the muscular layer (M), Kv1.3 labeled nerves (N). The serous membrane, a loose connective tissue which lines M, covered by peritoneal mesothelial cells was negative for both channels.

Fig. 7. Kv1.3 and Kv1.5 expression in human fetal epithelial tissues. Kv1.3 and Kv1.5 expression was analyzed in consecutive slices. A and B, Small intestine (200X); N, nerve (myenteric plexus); E, epithelium; M, muscular layer. C and D, Kidney (100X); PCT, proximal convoluted tubule; DCT, distal convoluted tubule; RC, renal corpuscle; CD, collecting duct. E and F, Liver (200X); PV, portal vein. G and H, Lung (200X); E, epithelium. I and J, Skin (200X); ED, epidermis; M, muscle; N, nerve.

Fig. 8. Kv1.3 and Kv1.5 expression in endocrine tissues. Kv1.3 and Kv1.5 expression was analyzed in consecutive slices in adrenal gland (A and B) and pancreas (C and D). A and B, adrenal cortex (200X). At 8-weeks of fetal development adrenal gland is mostly formed by the fetal type of adrenal cortex. C and D, pancreas (200X). At this early age, pancreatic acini are not yet formed and only pancreatic ducts (PD) from the exocrine pancreas are seen. Arrows in panel C indicates nerve (N).

Kv1.3 and Kv1.5 heavily stained different parts of the fetal kidney (Fig. 7 C and D). Accordingly, proximal (PCT) and distal (DCT) tubules, as well as collecting ducts (CD) stained for both channels. In contrast, renal corpuscles (RC) were less stained, especially for Kv1.5. During the early weeks of fetal development, the liver is a primary hematopoietic organ [32]. Therefore, both Kv1.3 and Kv1.5, which are mainly expressed in leukocytes and precursors [6], clearly stained hepatic structures (Fig. 7 E and F). During the embryo to fetal transition period during the 7th-17th weeks, the lung is in a pseudoglandular phase. The bronchioles, respiratory bronchioles, and alveolar ducts are formed by repeated dichotomous branching of lung buds. At this stage, Kv1.3 and Kv1.5 stained epithelial structures (Fig. 7 G and H). Figure 7 I and J show skin sections and nearby structures. While both Kv1.3 and Kv1.5 labeled the epidermis (ED), Kv1.3 (panel I) was also expressed in muscle (M) and nerve (N). However, while Kv1.5 heavily stained muscle (M), it was absent from nerve (panel J).

Figures 2 and 3 show that the pituitary gland (hypophysis, an active endocrine tissue) was strongly stained for both channels. Therefore, we further analyzed two heavily stained endocrine organs shown in Fig. 4, which were the adrenal gland (AG) and pancreas (PA). During this early stage of fetal development, the fetal adrenal cortex comprises most of the adrenal gland. Figure 8 A and B show that Kv1.3 and Kv1.5 stained the adrenal cortex specimens. On the other hand, during the embryo to fetal transition, the pancreas is an actively developing organ. While pancreatic acini are still not formed, the pancreatic ducts (PD) are visible and are surrounded by primitive mesenchymal cells (Fig. 8 C and D). Again, Kv1.3 and Kv1.5 notably stained duct epithelia. However, nerve structures were only labeled by Kv1.3.

Discussion

Our study is the first to address the expression of voltage-dependent K⁺ channels Kv1.3 and Kv1.5 during human fetal development. Both channels, which have been shown to be involved in processes associated with K⁺ transport, proliferation, differentiation and immune system physiology, were described in epithelia, muscle and nerve [6, 8, 10, 12, 13, 16, 23, 34]. Our results demonstrate that while Kv1.3 was localized to the central and peripheral nervous systems, Kv1.5 was more prominent in muscle. In addition, Kv1.5 was strongly expressed in the central but not peripheral nervous system.

Another important finding of our study was the strong expression of Kv1.3 and Kv1.5 in endocrine glands, such as the hypophysis, adrenal and pancreas.

Although widely present throughout the nervous system, Kv1.3 is prominent in oligodendrocytes, hippocampus and olfactory bulb [7, 35]. In fact, Kv1.3 knockout mice express an altered olfactory phenotype [36]. Our data confirm that Kv1.3 was concentrated in structures associated with the sense of smell such as the nasal fossa epithelium. The retina was other major Kv1.3 stained tissue. During embryonic development, the retina originates from the developing brain and contains several layers of neurons interconnected by synapses [37]. Within the brain, the trigeminal sensory ganglia and the pituitary gland also strongly stained for Kv1.3. Additionally, Kv1.5 also stained all of these organs. To what extent sensory organs express high levels of Kv1.3 and Kv1.5 is not known. However, the 8-10 week human fetal midbrain is undergoing important developmental changes. The vestibular system, which registers head and body motion, as well as gravity, starts developing at this stage. Just before this stage, the sensitivity to touch is first manifested by a series of protective movements in the 6 week embryo [30]. Electroencephalograms *in utero* have been used to detect brain waves and motor activity in several muscles and organs. From this early date, evidence has shown that skin sensitivity quickly extends to the genital area, palms of the hands, and soles of the feet [38]. These areas will have the greatest number and variety of sensory receptors in adults [30, 38]. In this sensory scenario, the most current research points to new and yet undetermined roles for olfaction *in utero*. The nasal chemoreceptive system is comprised of the four following subsystems: the main olfactory, the trigeminal, the vomeronasal, and the terminal system, which provide complex olfactory inputs to the fetus [39, 40]. Many of these organs were heavily stained for Kv1.3 and Kv1.5.

At 8-10 weeks, every organ of the fetus is in place, the hypothalamic nuclei begin to differentiate, undergoing neuronal precursor's proliferation and migration, and endocrine tissues express Kv1.3 and Kv1.5. However, the function of many hormones is still under research. Thus, although the role of fetal T3 and T4 thyroid hormones is not clear and thyroid regulation develops during the last months, the inactive reverse T3 (rT3) is higher in fetal tissues than in adults [41]. Hypothyroidism, which is commonly associated with atrial fibrillation, triggers a notable Kv1.5 abrogation [27, 28]. While the cardiopathy associates to Kv1.5 malfunctions, the endocrine disease onsets in neonates due to the deletion

of the Kv1.5-partner *kcne2*, which impairs channel expression [19, 28, 29]. In addition, fetal growth factors, such as Insulin Growth Factor 1 seem to appear later in development (at 12 weeks), but their role during the early stages of fetal development is not clear [42]. In this context, Kv1.3 expression has been shown to be related to glucose metabolism and the actions of insulin [9, 18], and previous studies have indicated that single-nucleotide polymorphisms in the promoter region of Kv1.3 are associated with impaired glucose tolerance and reduced insulin sensitivity [18]. Therefore, the Kv1.3 channel represents a candidate gene for type 2 diabetes.

Both Kv1.3 and Kv1.5 were expressed in fetal muscle. However, Kv1.3 was much less abundant. Differential expression of several K⁺ channels together with myogenic markers during development in muscle has been documented [21, 23]. However, no previous publications have examined the developmental expression patterns of Kv1.3 and Kv1.5. While myogenesis triggers an increase of Kv1.5, myoblast proliferation induces expression of both channels [10, 43]. However, only Kv1.5 seems to play a role in this process [10]. Alternatively, increasing evidence has implicated Kv1.3 in glucose metabolism [9, 18].

An important finding of the present study was the strong expression of Kv1.3 and Kv1.5 in fetal liver. Adult hepatocytes do not express Kv1.3 [13]. However, the fetal liver functions as a hematopoietic tissue during early

gestation and is characterized by the emergence and rapid expansion of progenitor cells for all types of leukocytes [32, 44]. Lymphoid-hematopoietic progenitor cells migrate from the intraembryonic mesenchyme to the fetal liver and relocate in bone marrow and thymus during late gestation [32, 44]. Although limited, the immune system has a defined repertoire of K⁺ channel genes. Leukocytes express, Kv1.3, Kv1.5 and several regulatory subunits [13, 14, 45, 46]. Moreover, heteromeric Kv1.3 and Kv1.5 complexes fine-tune the immune responses [16]. Our results are in accordance with the presence of leukocyte progenitors in human fetal liver at approximately the 8-10th week of gestation.

In summary, this study is the first to show the expression of Kv1.3 and Kv1.5 during fetal development in humans. Both channels are highly expressed in sensory, epithelia and endocrine tissues. The ontogenetic regulation of these channels may play a crucial role for the development of the mature Kv channel pattern in human.

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