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MINIREVIEWS

Sirtuins in B lymphocytes metabolism and function

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Abstract

Sirtuins (SIRTs) are NAD^+-dependent histone deacetylases and play a role in virtually all cell biological processes. As SIRTs functions vary according to their subtypes, they can either activate or inhibit signaling pathways upon different conditions or tissues. Recent studies have focused on metabolic effects performed by SIRTs in several cell types since specific metabolic pathways (*e.g.*, aerobic glycolysis, oxidative phosphorylation, β-oxidation, glutaminolysis) are used to determine the cell fate. However, few efforts have been made to understand the role of SIRTs on B lymphocytes metabolism and function. These cells are associated with humoral immune responses by secreting larger amounts of antibodies after differentiating into antibody-secreting cells. Besides, both the SIRTs and B lymphocytes are potential targets to treat several immune-mediated disorders, including cancer. Here, we provide an outlook of recent studies regarding the role of SIRTs in general cellular metabolism and B lymphocytes functions, pointing out the future perspectives of this field.

Key words: B cells; Metabolic sensors; Histone deacetylases; Cancer

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Core tip: Current studies have focused on understanding which intracellular molecules coordinate the metabolic flux within the cells. In addition to metabolism, sirtuins play a role in virtually all cell biological processes, but they have not been properly described in B lymphocytes function and metabolism, despite the importance of these immune cells in health and disease. Here we discuss studies that associate sirtuins and B lymphocytes, highlight the gaps found in the literature and point out the future research

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INTRODUCTION

Over the past decade, studies have focused on understanding which intracellular molecules coordinate the metabolic flux within the cells, mainly those belonging to the immune system[1-3]. Under activation, immune cells suddenly shift their metabolic profile to achieve their cell fates. These changes provide sufficient energy and generate a diversity of metabolic intermediates to allow rapid proliferation, perform specific functions and thus successfully combat the inflammatory insult[4]. Hence, immune cells are metabolically active populations which quickly respond to external signals (cytokines, chemokines, hormones, growth factors) to meet their bioenergetic demand.

However, the role of the metabolic sensors has been evaluated in only a few immune cell types, such as T cells, macrophages and dendritic cells[1,2]. The B lymphocytes are the main components of humoral responses, responding to both specific and non-specific antigens, producing antibodies after differentiation into plasmablasts/plasma cells [antibody-secreting cells (ASCs)], and generating immunological memory after antigen re-exposure[5]. The functional diversity of B lymphocytes (protection, regulation, effector function, memory) makes these cells essential during immune responses[6]. Thus, any changes in B cell development or function is sufficient to develop several diseases (immunodeficiency, autoimmunity, cancer). Despite their great importance in immune responses, the means by which the metabolic sensors act on the development and function of B lymphocytes has been sparsely evaluated[7].

The molecular sensors have evolutionarily developed within the cells. They converge a plethora of environmental signals that induce abrupt metabolic changes, leading the cells to achieve different fates (*e.g.*, differentiation, activation, anergy, autophagy or cell death)[3]. The most studied metabolic sensors include the mammalian (or mechanistic) target of rapamycin complex 1 (mTORC1), AMP-activated protein kinase (AMPK), hypoxia-inducible factor 1-alpha (HIF-1α), c-Myc protein, peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element-binding proteins (SREBPs)[3]. Recently, another protein family has been pointed out as an important metabolic sensor, the sirtuins (SIRTs). These nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases or adenosine diphosphate-ribosyltransferases are not only related to cell metabolism, but also to cell proliferation, survival, senescence, stress, gene stability, ribosomal DNA recombination and epigenetic regulations[8]. The wide range of SIRTs functions is due to their variable distribution within cells (cytoplasm, nucleus, and mitochondria) and highlights the importance of these proteins in cell biology. Here we briefly describe the SIRTs structure, distribution and functions, outline their role on general metabolism aspects, characterize the origin and development of B lymphocyte subtypes, and provide an outlook of recent studies regarding the role of SIRTs on metabolism, growth and function of B lymphocytes, pointing out the gaps that need to be filled in the next few years.

SIRTUINS

Initially identified in *Saccharomyces cerevisiae* as lifespan yeast proteins[9], it is now known that SIRTs constitute a highly conserved protein family among bacteria, plants and mammals[10,11]. The founding member of this family was discovered through a spontaneous mutation that caused sterility in yeast. The mutation reduced the transcription of the silent mating-type loci HML and HMR, later called as Mating-type Regulator 1 (MAR1) and currently named as Silencing Information Regulator 2 (SIR2)[12]. Twelve years later, it was identified that the SIR2-induced silencing of the mating-type loci in yeasts was associated with low levels of histone acetylation at the

N-terminal lysine residues of H4 histones[13]. Consistent with this finding, the overexpression of SIR2 promoted significant histone hypoacetylation and consequently extended yeast lifespan[14]. Thus, the SIRs (or SIRTs in humans/mice) were first classified as class III histone deacetylases.

The SIRTs activity is controlled by the intracellular NAD^+/NADH ratio, being activated when NAD^+ levels are increased[15,16]. SIRTs catalyze the acetyl groups removal of acetylated lysine-containing proteins to generate a deacetylated protein, free nicotinamide and a unique acetyl-ADP-ribose (O-ADP-ribose) metabolite, which is formed by the transfer of the acetyl group into the ADP-ribose fraction of NAD^+[17,18].

Although SIRTs were originally identified as histone lysine deacetylases, several other biological processes over numerous non-histone substrates have also been described. Hence, some SIRTs subtypes may play roles as deacetylase, desuccinylase, demaloynylase, deglutarylase, long-chain deacylase, lipoamidase or ADP-ribosyl transferase enzymes[19,20]. It has also been shown that SIRT isotypes display different specificities for ε-N-acyllysine post-translational modifications (PTM), an essential epigenetical modification process. Moreover, SIRTs affinities for various substrates still need to be investigated[21,22]. It is also important to emphasize that all enzymatic activity performed by SIRTs are dependent on the NAD^+ availability, thus reflecting the cellular metabolic status. Consequently, SIRTs have also been classified as metabolic sensors.

SIRTs have a conserved catalytic core formed by two domains which are responsible for catalyzing the transference of an acetyl group from a protein to a NAD^+ molecule. One domain is a large and well-conserved Rossman-fold domain, characteristic of NAD^+/NADH binding proteins, which accommodates NAD^+[23,24]. The other domain is smaller, less conserved and contains a zinc binding site. Although zinc does not actively participate in the deacetylation process, it plays a role in the structural integrity required for the reaction, since the SIRT deacetylase function is abolished when the zinc binding site is mutated[25]. There are also four polypeptide chains linking both larger and smaller domains, forming a cleft in which the substrates, NAD^+ and acetyl-lysine-containing protein bind on opposite sides. These four connecting polypeptide chains vary in size and sequence according to different SIRT isotypes, and such diversity may interfere with enzymatic activity, protein location and substrate specificity[10,26].

In yeasts, four SIRs have been identified (SIR1-4), whereas in humans and mice seven homologs (SIRT1-7) have been described[27]. SIRTs can be divided according to a specific terminology based on their structural sequence: SIRT1, 2 and 3 (class I), SIRT4 (class II), SIRT5 (class III) and SIRT6 and 7 (class IV)[28]. Despite the high structural similarity among the SIRTs, each one presents unique features regarding their enzymatic activities, cellular sublocations, and molecular targets as shown in Table 1. Therefore, the classification based on cellular sublocation has been most widely used, being SIRT1, 3, 6 and 7 classified as nuclear (SIRT1 may also be found in cytoplasm), SIRT2 as cytoplasmic (but it can also be found in nucleus) and SIRT3, 4 and 5 as mitochondrial proteins[29].

In summary, SIRTs were formerly described as histone deacetylases, but their enzymatic capacity has now been extended to several other non-acetylated substrates. Moreover, their ubiquitous distribution within cells and different tissues virtually expands the function of SIRTs for all cellular biological activities. The dependency of SIRTs on NAD^+ make them critical metabolic sensors that control several metabolic processes which will be described below.

SIRTUINS AND METABOLISM

As important metabolic sensors, studies have demonstrated that SIRTs modulate the gene expression, PTMs, and activity of key metabolic enzymes associated to glycolysis, oxidative phosphorylation (OXPHOS), glutamine metabolism, β-oxidation and fatty acid synthesis (Figure 1)[30,31].

Glucose is the primary cell metabolic fuel and provides several intermediates to other biosynthetic reactions in all mammalian cells, plants and many microorganisms. Glucose is transported into cells *via* the high-affinity glucose transporters (GLUT)[32]. While adipose tissue, liver, and muscle depend on the GLUT4 isoform, both T and B lymphocytes rely on GLUT1[33]. Moreover, other non-immune cells may express sodium-glucose linked transporters (SGLT), which import both glucose and sodium ions into the cell[34]. The glucose may be addressed to several metabolic pathways within a cell but is primarily destined for glycolysis.

Glycolysis is a cytosolic metabolic pathway in which glucose is converted into two pyruvate molecules. The pyruvate can be either used to synthesize acetyl-CoA and

Table 1 Sirtuins localization and function

Sirtuin	Localization	Enzymatic activity	General functions	References[1]
SIRT1	Nucleus Cytoplasm	Deacetylase Deacylase	Metabolism Mitochondrial biogenesis Cellular stress Chromatin regulation Cell differentiation	[30]
SIRT2	Cytoplasm Nucleus	Deacetylase Demyristoylase ADP-ribosylase Deacylase	Cell cycle Cell differentiation Metabolism Tumor suppression	[97]
SIRT3	Mitochondria	Deacetylase Decrotonylase Deacylase	Metabolism Mitochondrial biogenesis Antioxidant activity	[98,99]
SIRT4	Mitochondria	ADP-ribosylase Lipoamidase Deacetylase Deacylase	Tumor suppression Metabolism Tumor suppression	[100]
SIRT5	Mitochondria	Desuccinylase Deacylase Demalonylase Deglutarylase Deacetylase	Metabolism	[101]
SIRT6	Nucleus	Deacylase Deacetylase ADP-ribosylase	DNA repair Metabolism Inflammation	[102]
SIRT7	Nucleus	Deacetylase Deacylase	Ribosome biogenesis Tumor promotion Metabolism	[103]

ADP: Adenine diphosphate; SIRT: Sirtuin.

fuel the tricarboxylic acid (TCA) cycle or converted into lactate. Since lactate production usually occurs upon oxygen deprivation, this metabolic pathway is referred to as "lactic fermentation." Nevertheless, in B lymphocytes and other cells, the lactate production can occur even in the presence of oxygen, and it is termed "aerobic glycolysis" or Warburg effect[35]. For each molecule of glucose produced during glycolysis, there is a net sum of two units of adenosine triphosphate (ATP), an energy storing molecule[36]. As stated earlier, SIRTs play several roles in regulating glycolysis. SIRT1, 3 and 6 have been shown to suppress glycolysis while enhancing β-oxidation. SIRT1 activates the peroxisome proliferator-activated receptor γ coactivator-1α (PGC1-α) and inhibits HIF1-α, essential inducers of OXPHOS/β-oxidation and glycolysis, respectively. Mice lacking SIRT3 increase the reactive oxygen species (ROS) production, which in turn stabilizes HIF-1α inside the cell nucleus, enhancing glycolysis[37]. SIRT3, in turn, inhibits superoxide dismutase 2 (SOD2) enzyme to suppress the ROS-mediated stabilization of HIF1-α. SIRT6 acts as a co-repressor of HIF1-α and also inhibits c-Myc, another crucial glycolytic regulator which is also associated with glutamine metabolism[37]. On the other hand, SIRT5 can increase glycolysis by activating the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through demalonylation reactions[38]. Altogether, these results show that several SIRTs control positive and negatively the glycolysis. However, further studies are needed to understand why similar proteins have opposite or overlapped functions in regulating the glucose within cells.

One study showed that during the late acute inflammatory response, there is a metabolic shift from glycolysis towards OXPHOS in several immune cells types, characterized by lower expression levels of GLUT1, which was shown to be dependent on SIRT1 and SIRT6[39]. Furthermore, knockdown of these proteins results in a decreased GLUT1 expression in human monocytic cell lines activated with LPS, indicating an essential metabolic role of SIRTs during inflammatory responses[39]. We speculate that SIRTs, as regulators of glycolysis, might affect the GLUT1 expression among immune cell subpopulations. However, this information is still understudied and requires further investigation.

If the pyruvate is not converted into lactate, it can be oxidized into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex in the mitochondria, entering the TCA cycle. Acetyl-CoA combines with oxaloacetate to originate citrate and a series of following reactions take place to give rise to important products: NADH and $FADH_2$. Most non-proliferating and terminally differentiated T cells (*e.g.*, naïve and memory T cells), as well as resting B lymphocytes, use the TCA cycle to generate NADH and $FADH_2$ coenzymes,which transfer electrons to fuel OXPHOS[40]. The OXPHOS produces thirty six molecules of ATP per mol of glucose[41], and it is the most efficient method for generating energy from different metabolic intermediates, such as glucose, fatty acids or amino acids, although the slowest one[42].

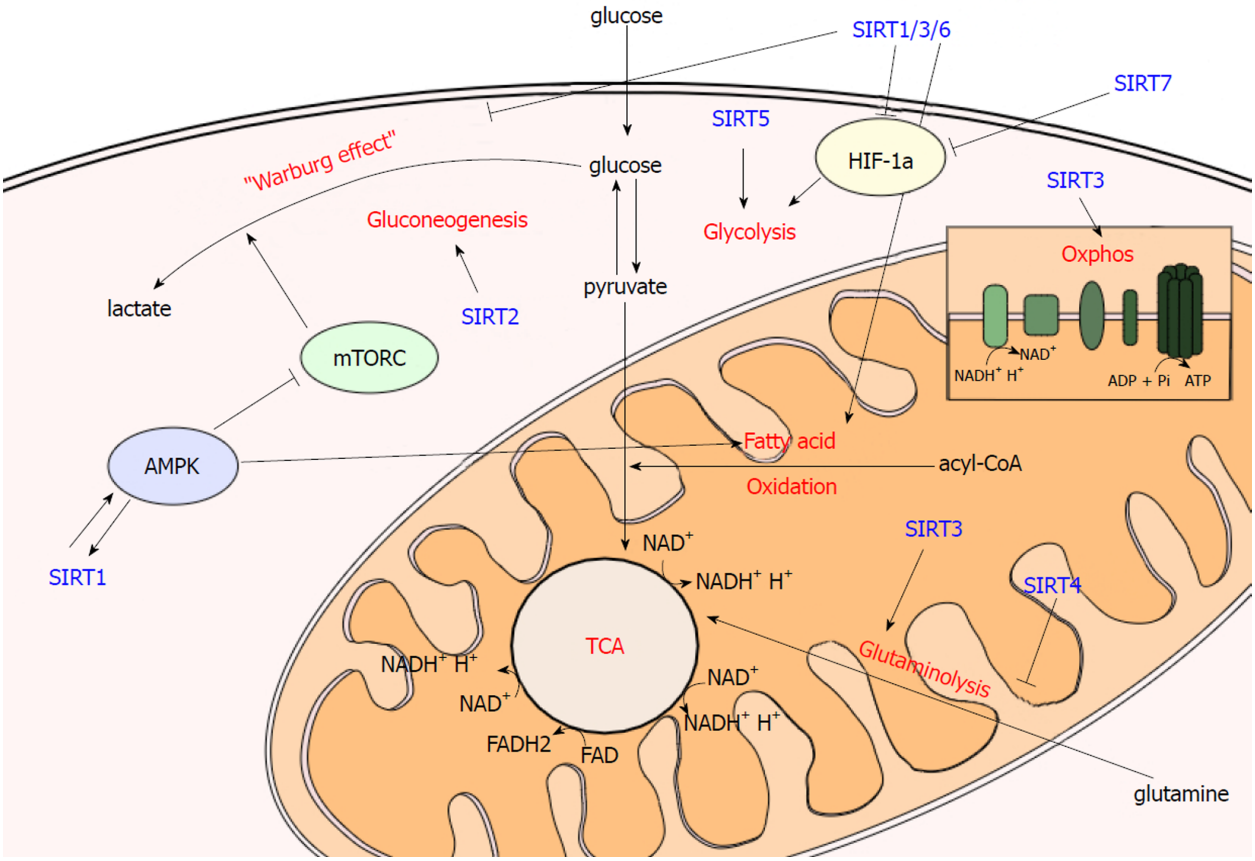


Figure 1 Sirtuins and metabolism. SIRTs are metabolic sensors that modulate a variety of metabolic pathways, including glycolysis (Warburg effect), gluconeogenesis, fatty acid oxidation, glutaminolysis, TCA cycle and OXPHOS. SIRTs 1, 3 and 6 restrain the glycolytic pathway through HIF-1α inhibition or direct effects. SIRT3 upregulates OXPHOS pathway by enhancing the activity of the mitochondrial complexes I, II and III and dampening ROS production. SIRT1 is also able to increase the fatty acid oxidation by activating PPAR-α and PGC1-α, while SIRT3 upregulates the fatty acid oxidation upon caloric restriction conditions. SIRT2 induces gluconeogenesis. SIRT3 and 4 activates and inhibits, respectively, the glutaminolysis by regulating the GDH activity. SIRT5 increases glycolysis by increasing the activity of the GAPDH enzyme. SIRT7 can also repress HIF-1α and therefore inhibit transcription of glycolytic genes. At last, SIRT1 performs a positive feedback loop with AMPK, since AMPK rises NAD^+ levels in cells, which in turn enhances SIRT1 activity and lastly leads to AMPK activation. AMPK suppresses glycolysis *via* mTORC1 inhibition and promotes fatty acid oxidation. In the Figure, SIRTs are represented based on their functions only and not by localization. AMPK: AMP-activated protein kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GDH: Glutamate dehydrogenase enzyme; HIF-1α: Hypoxia-inducible factor 1-alpha; mTORC1: Target of rapamycin complex 1; NAD^+: Nicotinamide adenine dinucleotide; OXPHOS: Oxidative phosphorylation; PGC1-α: Proliferator-activated receptor γ coactivator-1α; PPAR-α: Peroxisome proliferator-activated receptor alpha; ROS: Reactive oxygen species; SIRTs: Sirtuins; TCA: Tricarboxylic acid.

It is described that SIRT3 deacetylates several enzymes related to the TCA cycle, enhancing their activities. SIRT3 targets isocitrate dehydrogenase 2 (IDH), which catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate, and acetyl-coenzyme synthetase 2 that provides acetyl-CoA to the TCA in a PDH-independent manner[40]. SIRT1 has shown to be an activator of acetyl-CoA synthetase 1, restoring acetyl-CoA levels[43].

Fatty acids and glutamine also supply the TCA cycle and OXPHOS by additional metabolic reactions such as β-oxidation and glutaminolysis, respectively[44]. As stated above, SIRT6 was described as an important regulator of c-Myc activity. c-Myc has been associated with glutamine metabolism by activating the glutaminase 1 (GLS1), which converts glutamine to glutamate. SIRT3, in turn, increases the activity of glutamate dehydrogenase (GDH), responsible for converting glutamate into α-ketoglutarate, while SIRT4 inhibits GDH activity[31]. SIRT4 acts as a tumor suppressor protein, being able to inhibit mitochondrial glutamine metabolism by repressing GDH activity (Figure 1).

Regarding β-oxidation, SIRT1 has been shown to increase this pathway activity by activating both PPAR-α and PGC1α, thus promoting the expression of downstream targeted genes which are related to increased use of lipids. At the same time, SIRT1 inhibits lipid synthesis through deacetylation of SREBP-1c or *via* suppression of PPAR-γ[45]. Upon caloric restriction, SIRT3 activates long-chain Acyl-CoA dehydrogenase (LCAD) and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), promoting β-oxidation and ketogenesis, respectively. Also, SIRT3 also enhances cellular respiration by enabling mitochondrial complexes I, II and III and decreasing ROS production, given it stimulates the activity of the SOD2. SIRT4 dampens the

transcription of genes underlying β-oxidation, such as PPAR-α, whereas SIRT6 is thought to repress the transcription of fatty-acid synthesis-related genes[45].

Interestingly, SIRTs can play their role along with another critical metabolic sensor, the AMPK. AMPK is usually activated by increasing intracellular calcium influxes or AMP/ATP ratio in the cells. This kinase inhibits the activity of mTOR, a member of the PI3Ks protein kinase family which plays a central role in upregulating glycolysis as well as protein synthesis, energy balance, cell proliferation and survival[3]. Therefore, AMPK inhibits glycolysis and promotes both β-oxidation and OXPHOS in several cell types[3,46,47]. AMPK can also act on metabolic reprogramming through transcriptional and PTM as observed in SIRTs[48,49]. Studies show that SIRT1 forms a positive feedback loop with AMPK: AMPK increases NAD^+ levels in cells, which in turn enhances SIRT1 activity and lastly leads to AMPK activation[50].

Although the role of SIRT2 in metabolism has not been appropriately investigated, it induces gluconeogenesis in the adipose tissue *via* PGC1α and FOXO1 activation[51,52]. FOXO1 is a vital coordinator of longevity, tumor suppression, metabolism and cell growth[53]. Gluconeogenesis is a metabolic pathway in which glucose is synthesized from non-carbohydrate precursors (*e.g.*, lactate, glycerol and some amino acids) being activated in some specialized tissues under glucose deprivation states. Gluconeogenesis has not been described in immune cells, but a recent study showed that memory T cells upregulate the gluconeogenesis-related enzyme Pck-1 to increase gluconeogenesis activity[54]. SIRT7 expression and function in immune cells have not been well characterized. However, SIRT7 regulates low glucose-induced cellular stress by uncoupling rRNA synthesis and enabling energy storage in HEK293T cells[55]. Also, SIRT7 can also repress HIF-1α (Figure 1) and therefore inhibit the transcription of glycolytic genes in many cell lineages such as Hep3B, HeLa, HEK293T and MDA-MB-231[55].

Briefly, SIRTs may either activate or inhibit metabolic pathways depending on their isotypes, localization and cellular activation status. Moreover, SIRT1 is the most well-described isotype and regulates several metabolic pathways, whereas SIRT2 and 7 lack metabolic descriptions in immune cells.

B LYMPHOCYTES

B lymphocytes have a pivotal role in adaptive immune responses through cytokine secretion, antigen presentation to T cells, as well as by their unique ability to produce antibodies after differentiating into ASCs[56-58].

Early in life, B lymphocytes are produced in the fetal liver, and after birth, they are generated in the bone marrow (BM) throughout life[5] (Figure 2). In addition, current studies have found that B lymphocytes can also develop from the gut shortly after birth and the resident microbiota plays a crucial role in increasing the antigen receptor repertoire of these cells[59].

The first B-cell lineage-committed progenitors are derived from hematopoietic stem cells and known as pre-pro B lymphocytes. These cells express B lymphocyte-specific surface proteins such as B220 (or CD45R)[60]. Subsequently, these cells undergo μ heavy chain somatic recombination through rearrangement of V(D)J gene segments to assembly the pre-BCR, becoming pro-B lymphocytes[61]. In this stage, cells start to express the CD19 coreceptor under the control of the transcription factor Pax5[62]. The pre-BCR is a transitory complex consisting of a successful V(D)J recombination, a surrogate light chain and two intracellular signaling proteins (Igα and Igβ); pre-BCR is expressed in the surface of pre-B lymphocytes[63,64]. If the pre-BCR results from a nonproductive V(D)J recombination process, then the pre-B lymphocyte development stops and the cell undergoes apoptosis[65]. The correct signaling through the pre-BCR promotes intracellular changes that block second allele recombination, in a process termed as allelic exclusion[66]. Furthermore, the proper BCR signaling induces somatic recombination of κ or λ light chains through the rearrangement of VJ segments, allowing the BCR assembly[64,65]. Upon reaching the immature stage, B lymphocytes express a functional BCR as a surface IgM protein. Finally, the immature B lymphocytes leave the BM towards the spleen where further developmental steps occur, by the time they are ultimately differentiating into follicular (FO) or marginal zone (MZ) B lymphocytes.

Before B lymphocytes differentiate into FO or MZ B cells they go through three transitional stages (T1, T2 and T3)[67], which are quite different between human and mouse[68]. T1 and T2 B lymphocytes give rise to mature B lymphocytes, whereas the development and function of T3 cells remains unclear[69]. The role of these transitional B lymphocytes is to allow the second round of regulatory checkpoint aiming to decrease potential cell autoreactivity.

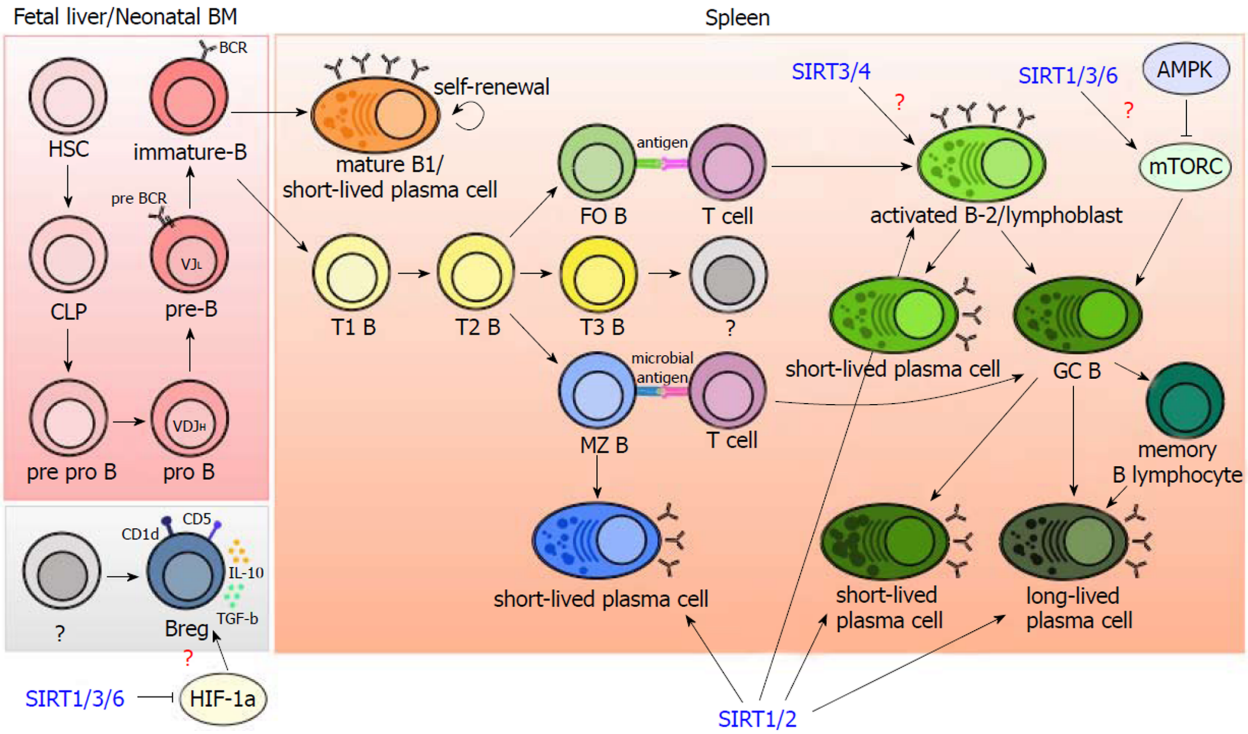


Figure 2 Sirtuins and B lymphocytes. B lymphocyte development begins in the fetal liver or bone marrow and continues in the periphery. B-1 and B-2 B lymphocytes differentiate into ASCs by distinct pathways. The origin of Breg cells is still not precise. Despite the role of SIRTs in healthy B lymphocytes have not been adequately investigated, some studies have described some functions in a disease context. SIRT1 and 2 inhibition are essential to treat CLL, although other studies show that SIRT1 is important at some point of ASCs differentiation. Moreover, since AMPK-mTORC1 axis regulates GC reactions and antibody production, it suggests that SIRT1 (or SIRT3/4) also coordinate this process. Glutaminolysis was shown to be an essential metabolic pathway to enhance proliferation of Burkitt lymphoma-derived B lymphocytes. Thus, SIRT3 and 4 might play roles in the pathogenesis by activating or inhibiting, respectively, the GDH activity. Additionally, HIF-1α was shown to be essential to IL-10-producing Bregs development, suggesting that SIRT1/3/6 are downregulated in these populations. The question marks indicate speculative roles of SIRTs. AMPK: AMP-activated protein kinase; ASCs: Antibody-secreting cells; B: B lymphocytes; BCR: B cell receptor; Breg: B regulatory lymphocytes; CLL: Chronic lymphocytic leukemia; CLP: Common lymphoid progenitor; FO B: Follicular B lymphocytes; GC: Germinal center; GDH: Glutamate dehydrogenase enzyme; HIF-1α: Hypoxia-inducible factor 1-alpha; HSC: Hematopoietic stem-cell; mTORC1: Target of rapamycin complex 1; MZ: Marginal zone; SIRTs: Sirtuins; T1/T2/T3: Transitional stages 1, 2 and 3, respectively, of B lymphocytes.

Although MZ B lymphocytes are usually associated to T-independent B lymphocyte responses, one study showed that they could respond to T lymphocytes, differentiate into germinal center (GC) cells and subsequently in ASCs and memory B lymphocytes[70]. However other studies are needed to confirm this observation.

FO B lymphocytes can recirculate and migrate to follicles within secondary lymphoid organs. They are located nearby follicular $CD4^+$ T cells, allowing bidirectional cooperation during T-dependent B lymphocytes responses. However, FO B lymphocytes are also found within the BM in small clusters, where they respond to bloodborne pathogens in a T-independent manner[71]. FO B lymphocytes are the primary source of memory B lymphocytes and plasmablasts that can terminally differentiate in long-lived plasma cells and synthesize large amounts of antibodies.

Another possible fate of immature B lymphocytes is to become B-1 cells, which are able to self-renew in the periphery, populating the fluids from the pleural, peritoneal and intestinal cavities[72,73]. These cells produce antibodies independently of T cell assistance and are often referred to as innate mediators. An important role of B-1 cells is to synthesize polyspecific natural antibodies (IgM and IgA), which recognize several carbohydrate residues and rapidly respond to mucosal pathogens[57,73].

Another emerging B lymphocyte population are the regulatory B cells (Bregs). Similarly to the well-described regulatory T lymphocytes (Tregs), Bregs inherently produce IL-10 to control inflammatory responses, but also TGF-β and IL-35[74,75]. In addition, Bregs can induce apoptosis or anergy of T cells, suppress the differentiation of monocytes and dendritic cells and cooperate with Treg differentiation[74]. The origin of these cells is not well established, although it is known that conventional B lymphocytes can differentiate into Breg cells at all stages of development[76].

B lymphocytes are associated with several pathological conditions, such as autoimmunity, non-autoimmune inflammatory diseases, and cancer. In Systemic lupus erythematosus (SLE), for instance, larger amounts of autoantibodies able to recognize nuclear proteins are produced and may accumulate as immune complexes

in the joints, skin, kidney and serosal membranes, leading to a severe inflammatory status[77]. Moreover, chronic lymphocytic leukemia (CLL), the most common form of leukemia in western countries, is a type of cancer that arises from uncontrolled proliferation of B lymphocytes in lymphoid and non-lymphoid organs[78]. CLL is characterized by a poor outcome and reduced survival rates among the affected patients.

In summary, B lymphocytes are the powerhouses of humoral immune responses given their ability to secrete antibodies capable of neutralizing and opsonizing pathogens. Besides, B lymphocytes undergo multiple developmental steps to generate different subsets. Thus, given the complexity of B lymphocytes, it is reasonable to assume that these cells cooperate in maintaining homeostasis and that any putative changes in their function or maturation steps might contribute to the development of several pathologies.

SIRTUINS, B LYMPHOCYTES, AND METABOLISM

Few studies have focused on understanding B lymphocyte metabolism compared to other immune and non-immune cell types. It is described that in a resting state, B lymphocytes present higher rates of glycolysis when compared to T cells; upon activation, they increase both glycolysis and OXPHOS at similar rates[32]. However, only glycolysis but not OXPHOS was shown to be essential for LPS-activated B lymphocyte development, proliferation, and function[79]. When glycolysis is impaired *in vitro* at distinct steps, the proliferation of stimulated B lymphocytes and antibody secretion are strongly suppressed[32]. B lymphocytes lacking GLUT1 show significantly IgM and IgG production impairment in immunization models[32]. In addition to glycolysis, fatty acids are also produced *de novo* to support synthesis and expansion of membranes in plasma cells[80]. Concomitantly, fatty acids are essential to generate energy *via* β-oxidation. Since antibodies are glycoproteins, the metabolism of amino acids and glucose-derived intermediates are necessary during all antibody generation process[81].

The role of SIRTs in B lymphocytes has been described under pathological conditions and lacks information on healthy B lymphocytes. Recent studies indicate that SIRT1 regulates the immune response by delaying the onset of autoimmunity since nuclear-reactive autoantibodies were found in the sera of SIRT1-null mice. Moreover, these animals had deposits of immune complexes within the liver and the kidneys, indicating an autoimmune-like condition due to the lack of SIRT1[82]. Since a rapid and increased glycolytic activity is found under chronic B lymphocyte Activating Factor (BAFF)-exposure in B lymphocytes[32] to induce experimental autoimmunity, it is plausible to consider that SIRT1 counter-regulates the glucose pathway in healthy B lymphocytes. However, it is still not a matter of investigation.

Another study showed that both SIRT1 and 2 contribute to CLL pathogenicity. SIRT1 mRNA expression and protein levels were increased in B lymphocyte-derived cell lines from human patients with CLL compared to control healthy group[78]. Additionally, pharmacological inhibition of both SIRT1 and 2 using EX-527 and sirtinol, respectively, in PBMC cells from patients with CLL resulted in dose-dependent cytotoxicity, increased apoptosis rates and elevated mitochondrial ROS production[78]. These results indicate SIRTs as potential targets for clinical trials in patients affected by CLL and for other B lymphocyte-related conditions.

It has also been described that SIRT3 expression is reduced in CLL, leading to accumulation of ROS and induction of a Warburg-like metabolic pathway that supports the uncontrolled proliferation of B lymphocytes[83]. However, the underlying metabolic changes in these pathogenic B lymphocytes require further investigations.

Pan-histone-deacetylase inhibitors (HDACi) such as panobinostat have shown to be capable of reducing autoreactive plasma cell counts and autoantibodies in a mouse model for SLE[84]. However, immunological memory was not compromised after the treatment, given that the level of circulating memory B lymphocytes remained unaltered. Meanwhile, it is not possible to state whether SIRTs mediate these changes or if other histone deacetylases are more relevant in this context.

Another study suggests that the microRNA 34a(miR34a)-SIRT1-p65 axis is crucial for activating intestinal immune responses during chronic simian immunodeficiency virus (SIV) infection in rhesus macaques. It was described that miR-34a upregulation coupled with a downregulation of SIRT1 enhance the NF-κB activity in both IgA^+ and IgG^+ intestinal plasma cells, contributing to B lymphocyte hyperresponsiveness in chronic SIV-infected macaques[85].

The glutamine metabolism has been shown as an essential regulator of B lymphocyte proliferation and survival under glucose deprivation and hypoxia

conditions in P493 cells, a B lymphocyte-derived cell line to study Burkitt lymphoma[86]. Hypoxia is an essential feature found in tumor microenvironments. Since SIRT3 and SIRT4 activates and inhibits, respectively, the glutaminolysis pathway, these proteins are potential targets for future studies focusing on glutamine metabolism in normal or cancerous B lymphocytes. Interestingly, SIRT4 deletion in a mouse model for Burkitt-lymphoma resulted in increased tumor proliferation and mortality rates, indicating that SIRT4 might act as a tumor suppressor as described in other cell types[87]. However, the association among B lymphocytes, metabolism and SIRT3/4 remains to be clarified in the Burkitt-lymphoma context (Figure 2).

Breg metabolism characterization has not been described so far, although HIF-1α is essential to IL-10-producing B lymphocytes development[88]. Nevertheless, it is well established that Treg metabolism rely on mitochondrial OXPHOS[4]. SIRT1 has already been associated with the induction of FoxP3$^+$ Treg cells, although it is still under investigation[89]. Moreover, AMPK has also been described as an important molecular sensor to induce Treg differentiation[90]. Altogether, these results suggest that Breg and Treg have different regulators since HIF-1α is pro-glycolytic (Figure 2).

Recent researches indicate that mTORC1 activity is essential for the GC reactions, increasing the rate of somatic hypermutation and affinity maturation of isotype-switched B lymphocytes[91]. However, AMPK has also shown to induce terminally differentiated plasma cells and enhance antibody production[92], suggesting that SIRT1 might be downregulated in GC B lymphocytes and upregulated in plasma cells. Nevertheless, underlying mechanisms describing how this putative metabolic shift occurs during the process is unknown. Also, future studies should investigate whether SIRTs have distinct roles in differentiating plasma cells and memory B lymphocytes during GC reactions.

Importantly, B lymphocytes play substantial roles in the pathogenesis of metabolic (*e.g.*, obesity, cancer, diabetes, periodontal disease) and non-metabolic (SLE, reumathoid arthritis, graft-verus-host diseases, HIV infection) conditions[93-103]. Therefore, future investigations on the impact of SIRTs in B lymphocytes metabolism and function will provide potential alternatives on treating or dampening the progression of a wide range of illnesses.

In summary, the role of SIRTs in B lymphocytes remains under investigation, and the association with metabolic aspects is at the beginning of understanding. Further studies focusing on comprehension of SIRTs functions in the development and metabolism of B lymphocytes under homeostasis conditions must also be encouraged.

CONCLUSION

Altogether, these results show that SIRTs play roles in virtually all biological processes in cells but should be further evaluated in B lymphocytes since they are related to several homeostatic and pathologic responses. Moreover, several SIRTs isotypes have not been sufficiently investigated, and future studies are necessary to achieve a broader and more complete understanding of their functions in the cell metabolism. It is important to state that B lymphocytes have several cell subsets and the assessments regarding the role of metabolic sensors should be performed in specific B lymphocyte subpopulations. Thus, future investigations must answer whether or which SIRTs are important to each B lymphocyte subtype in both healthy and pathological states. The better comprehension of how metabolic sensors, especially SIRTs, control the development, metabolism, function, and lifespan of immune cells is therefore essential and suggests they may be valuable and potential pharmacological targets to treat several metabolic-related diseases.

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ORIGINAL ARTICLE

Basic Study

Localization of ATP-sensitive K$^+$ channel subunits in rat liver

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Author contributions: Zhou M did the main experiments, collected the data, and wrote the paper; Yoshikawa K and Miura M performed the experiment of primary culture for hepatic stellate cells and Kupffer cells; Akashi H and Suzuki R participated in treatment of animals; Li TS, Abe H, and Bando Y supervised the investigation and approved the manuscript for publication.

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Abstract

BACKGROUND

ATP-sensitive K$^+$ (K_{ATP}) channels were originally found in cardiac myocytes by Noma in 1983. K_{ATP} channels were formed by potassium ion-passing pore-forming subunits (Kir6.1, Kir6.2) and regulatory subunits SUR1, SU2A and SUR2B. A number of cells and tissues have been revealed to contain these channels including hepatocytes, but detailed localization of these subunits in different types of liver cells was still uncertain.

AIM

To investigate the expression of K_{ATP} channel subunits in rat liver and their localization in different cells of the liver.

METHODS

Rabbit anti-rat SUR1 peptide antibody was raised and purified by antigen immunoaffinity column chromatography. Four of Sprague-Dawley rats were used for liver protein extraction for immunoblot analysis, seven of them were used for immunohistochemistry both for the ABC method and immunofluorescence staining. Four of Wistar rats were used for the isolation of hepatic stellate cells (HSCs) and Kupffer cells for both primary culture and immunocytochemistry.

RESULTS

Immunoblot analysis showed that the five kinds of K_{ATP} channel subunits, *i.e.* Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B, were detected in liver.

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Immunohistochemical staining showed that Kir6.1 and Kir6.2 were weakly to moderately expressed in parenchymal cells and sinusoidal lining cells, while SUR1, SUR2A, and SUR2B were mainly localized to sinusoidal lining cells, such as HSCs, Kupffer cells, and sinusoidal endothelial cells. Immunoreactivity for SUR2A and SUR2B was expressed in the hepatocyte membrane. Double immunofluorescence staining further showed that the pore-forming subunits Kir6.1 and/or Kir6.2 colocalized with GFAP in rat liver sections and primary cultured HSCs. These K_{ATP} channel subunits also colocalized with CD68 in liver sections and primary cultured Kupffer cells. The SUR subunits colocalized with GFAP in liver sections and colocalized with CD68 both in liver sections and primary cultured Kupffer cells. In addition, five K_{ATP} channel subunits colocalized with SE-1 in sinusoidal endothelial cells.

CONCLUSION
Observations from the present study indicated that K_{ATP} channel subunits expressed in rat liver and the diversity of K_{ATP} channel subunit composition might form different types of K_{ATP} channels. This is applicable to hepatocytes, HSCs, various types of Kupffer cells and sinusoidal endothelial cells.

Key words: ATP-sensitive K^+ channel; Liver; Hepatic stellate cells; Kupffer cells; Sinusoidal endothelial cells; Rat

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Core tip: ATP-sensitive K^+ (K_{ATP}) channels have been found to be ubiquitously distributed in a variety of cell types and tissues, but the present study revealed that five kinds of K_{ATP} channel subunits are expressed in rat liver by applying western blot analysis, immunohistochemistry, and immunocytochemistry of rat liver tissue and primary cultured cells. These K_{ATP} channel subunits were not only localized in hepatocytes but also in sinusoidal cells, such as hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells. This was demonstrated using specific marker labelling, such as GFAP for hepatic stellate cells, CD68 for Kupffer cells, and SE-1 for sinusoidal endothelial cells. The wide diversity of K_{ATP} channel subunit compositions in rat liver might correspond to their various functions in hepatocytes and sinusoidal cells.

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INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels were found ubiquitously distributed in a variety of cells and tissues[1], such as pancreatic β cells[2-5], skeletal muscle[6], neurons and glial cells[7-9], kidney[10-13], and liver[14,15]. K_{ATP} channels close at high concentrations of intracellular ATP and open at low concentrations during ischemia[7,16-18], and couple altered energy metabolism with membrane potential[19,20]. K_{ATP} channels are hetero-octamers composed of two kinds of subunits: the four pore-forming subunit Kir6.x (Kir6.1 or Kir6.2), and the four regulatory subunit sulfonylurea receptor SUR (SUR1, SUR2A, or SUR2B)[21-24]. Their physiological and pharmacological functions depend on their subunit compositions[25-27]. In the hypothalamus, glucose homeostasis is controlled by glucagon and catecholamine, which are counter-regulatory hormones, and these functions are involved with K_{ATP} channels[26,28]. The liver is also an organ that directly takes part in glucose metabolism with changes in intracellular ATP (ATPi) concentrations during which K_{ATP} channels are quite sensitive. The mRNA of K_{ATP} channels in liver is detected by northern blot analysis[14], K_{ATP} channels play roles in regulating hepatocyte proliferation in primary rat hepatocytes[29]. However, there are no detailed references on the localization of K_{ATP} channel molecules and their functions in the liver with respect to hepatocytes, hepatic stellate cells (HSCs), Kupffer

cells, and sinusoidal endothelial cells (SECs). In addition to glucose metabolism, the liver has important functions in regeneration after injury and partial hepatectomy, as well as some functions of immune defense because of Kupffer cells. HSCs are localized between SECs and hepatocytes, and are activated during liver injury, producing many growth factors, chemokines, and cytokines, and also excessive extracellular matrix. They are also known as one of the reasons for hepatic fibrosis after liver injury recovery[30-33]. We are interested in clarifying the relationships between K_{ATP} channel subunits and hepatocytes, Kupffer cells, HSCs, and SECs in the liver during normal and pathological conditions.

Since there is no detailed information on the localization of K_{ATP} channel molecules in the liver with respect to different cells, we focused on the expression and localization of these K_{ATP} channel subunits in liver, and supply morphological data to help understand what functions they could play in parenchymal and non-parenchymal cells.

MATERIALS AND METHODS

Animals and tissue preparation

Sprague-Dawley and Wistar rats were supplied by CLEA Japan Inc. (Tokyo, Japan). This study was conducted according to the protocols of the Animal Research Committee of Akita University. We handled the animals according to the Guidelines of Akita University for Animal Experimentation. Rats used in this study were kept under constant environmental conditions in group cages (2-3 rats per cage) with 12 h light/dark cycles, and normal food and water were given.

Male SD rats (4-8 wk) under deep anesthesia were perfused through the left ventricle with cold Zamboni fixatives (2 g paraformaldehyde solved in 15 mL of saturated picric acid and Karasson Schwlt's phosphate buffer solution added to make 100 mL). Liver tissue blocks were taken and further fixed in the same fixative for 6 h at 4 °C, and then changed to 30% sucrose in PBS until they sank to the bottom. No-fixed liver tissue was also taken for GFAP immunostaining. Cryosections were cut 10 μm thick with a Leica CM1950 cryostat, thaw-mounted on glass slides (MAS-coated, Matsunami Industries, Kishiwada, Japan) and stored at -20 °C before use. Liver samples for immunoblot analysis were directly taken out without fixation, put into dry ice powder, and stored at -80 °C until use.

Preparation of hepatic stellate cells and Kupffer cells

Male Wistar rats (400-500 g body mass), maintained on a normal chow diet, were used. Rats were perfused *via* the portal vein in situ with pre-perfusion solution (Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 10 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES), 0.5 mM EGTA, 4.2 mM $NaHCO_3$, and 5 mM glucose; pH 7.2) for 5 min at 37 °C, followed by collagenase solution (HBSS) containing 10 mM HEPES, 5 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 0.5 mg/mL bacterial collagenase; pH 7.5) for 15 min at 37 °C at a flow rate of 15 mL/min. Liver digested by collagenase solution was excised, dispersed, and filtered through stainless mesh. Isolation of HSCs and Kupffer cells was performed based on cell size and density[34]. To eliminate most of the parenchymal cells, the cell suspension was centrifuged at 50 × g for 1 min. The supernatant was further centrifuged at 50 × g for 3 min. The supernatant of the second run was centrifuged at 400 × g for 5 min to pellet non-parenchymal cells. The non-parenchymal cells in the pellet were further centrifuged at 700 × g for 30 min in 25% (*v*/*v*) Percoll PLUS solution (GE Healthcare Bio-Sciences, Piscataway, NJ, United States). HSCs were enriched in the supernatant, and Kupffer cells were mostly enriched in the pellet with other types of cells. The supernatant enriched in HSCs was diluted with 2 volumes of Dulbecco's modified Eagle's medium (DMEM) and centrifuged at 400 × g for 5 min to wash out Percoll PLUS. All centrifugations were performed at 4 °C. The cell fractions enriched in HSCs and Kupffer cells were suspended in culture media (DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin), and then plated on glass-bottom dishes coated with poly-L-lysine (Matsunami Glass, Osaka, Japan). Twelve to sixteen hours later, the cells were washed to remove debris, and then used for immunocytochemistry.

Generation of SUR1 antibody

Rabbit anti-rat SUR1 antibody was generated by immunization with a synthetic 22-mer peptide, NH2-DSALVLSPAARNCSLSQECALD-(COOH) (Biologica Co. Nagoya, Japan), which corresponds to amino acid residues of 1038-1059 of rat SUR1 (GenBank Accession No. L40624). Antiserum was purified by peptide antigen immunoaffinity

column chromatography.

Western blot analysis

SDS/PAGE was performed as the procedure of Laemmli. Proteins were treated with modified sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol blue, and 10% 2-mercaptoethanol). After electrophoresis, proteins (10 μg/lane) were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (NENTM Life Science, Boston, MA, United States) using a semi-dry transfer unit (Hoefer TE70 series; Amersham Pharmacia Biotechnology, Little Chalfont, Buckinghamshire, England). After blocking with 5% BLOT-QuickBlocker (Chemicon International, Inc., Temecula, CA, United States) in PBS at room temperature overnight, immunoblotting was carried out by incubating the PVDF membranes with polyclonal goat anti-human Kir6.1 and/or Kir6.2 (Santa Cruz Biotechnology) and rabbit anti-rat SUR1, SUR2A and/or SUR2B antibody[35], each diluted to 1:1000, respectively, for 1 h at room temperature. Following thorough washing with PBS-T (PBS with 0.1% Tween-20), the PVDF membranes were then incubated with HRP-conjugated donkey anti-rabbit IgG (NA9340; Amersham Pharmacia Biotechnology, England), or HRP-conjugated donkey anti-goat IgG (AP180P), each diluted 1:3000. Signals were visualized with chemiluminescence detection reagents (Amersham Pharmacia Biotechnology, England), and PVDF membranes were exposed to XOMAT film (Eastman Kodak, Rochester, NY, United States) in the dark room. Negative controls and specificity verification of the SUR1 antibody were performed using the antigen peptide preabsorption test.

Immunohistochemistry

After air drying for 30 min, cryosections of liver were treated with 0.3% Tween-20 in PBS for 45 min at room temperature. Sections were blocked either with 5% normal rabbit serum or 5% normal goat serum for 30 min, and then incubated with goat anti-human Kir6.1 (Santa Cruz Biotechnology), goat anti-human Kir6.2 (Santa Cruz Biotechnology), and rabbit anti-rat SUR1, SUR2A, and/or SUR2B, each diluted at 1:500. After washing with PBS three times, the biotinylated rabbit anti-goat IgG, or biotinylated goat anti-rabbit IgG, were applied to sections. As a negative control, the immunoreactive solution was prepared without the primary antibody, and an absorption test was also confirmed by pre-incubation with peptide antigen.

To show whether the pore-forming subunits Kir6.1 and/or Kir6.2 are colocalized with the regulatory subunits SUR1, SUR2A and/or SUR2B in the liver, sections were incubated with a mixture of goat anti-human Kir6.1 and rabbit anti-rat SUR1, and/or anti-rat SUR2A, and/or SUR2B; or a mixture of goat anti-human Kir6.2 with rabbit anti-rat SUR1, SUR2A and/or SUR2B antibody, each diluted 1:500, respectively, for 12 h at room temperature. Sections were then incubated with a mixture of Alexa488-conjugated donkey anti-goat IgG (A21432; Molecular Probes, Inc., Eugene, OR) and Alexa594-conjugated donkey anti-rabbit IgG (A21207; Molecular Probes, Inc., Eugene, OR), each diluted 1:200.

To determine whether the K_{ATP} channel subunit molecules colocalize with HSCs, Kupffer cells and/or SECs, sections were incubated with a mixture of mouse anti-GFAP (marker of HSCs), or incubated with a mixture of mouse anti-CD68 (marker of Kupffer cells), and/or incubated with a mixture of mouse anti-SE-1 antibody (marker of SECs) and one of the K_{ATP} channel subunit antibodies, such as goat anti-human Kir6.1, goat anti-human Kir6.2, rabbit anti-rat SUR1, rabbit anti-rat SUR2A, and/or rabbit anti-rat SUR2B, respectively, each diluted 1:200. After thoroughly washing with PBS, the liver sections were then incubated for 30 min with a mixture of the appropriate fluorescent Alexa 488 or Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR) each diluted 1:200. Finally, the sections stained above were mounted with aqueous mounting medium (Thermo, Pittsburgh, PA, United States).

Primary cultured HSCs and Kupffer cells were fixed with Zamboni's fixatives for 5 min and then double immunofluorecent staining was carried out as (1) mouse anti-GFAP with one of the antibodies of the pore-forming subunits Kir6.1 and/or Kir6.2, and/or one of the antibodies of the regulatory subunits SUR1, SUR2A and/or SUR2B; (2) mouse anti-CD68 with one of the antibodies of goat anti-human Kir6.1, and/or Kir6.2, and rabbit anti-rat SUR1, SUR2A and/or SUR2B; (3) goat anti-human Kir6.1 or Kir6.2 with one of the antibodies of rabbit anti-rat SUR1, SUR2A, and/or SUR2B. After thoroughly washing with PBS, the dishes were incubated for 30 min with the appropriate fluorescent Alexa 488- or Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR) each diluted 1:200. Fluorescence image signals were acquired using an Olympus microscope (BX51; Tokyo, Japan) and a laser scanning confocal microscope (LSM 510; Carl Zeiss, Germany).

RESULTS

Expression of K_{ATP} channel subunits in rat liver

Immunoblot analysis demonstrated that Kir6.1, Kir6.2, SUR2A and SUR2B were expressed in rat liver (Figure 1A). For the anti-SUR1 antibody, immunoblot analysis showed that the molecular weight of SUR1 was about 100 kDa with some weak small bands below. After preincubation with immunizing peptide from which the antibody was raised, the SUR1 band disappeared (Figure 1B, +peptide).

Immunohistochemistry revealed that immunoreactivity for Kir6.1 was widely and weakly observed in hepatocytes in the hepatic lobules (Figure 2A), as well as in the endothelia of the central vein, the branches of the hepatic artery, and the portal vein at the portal area (Figure 2C). The intensity of immunoreactivity was gradually decreased along the liver plate from the central vein to the distal area (Figure 2A). Weak immunoreactivity for Kir6.1 was expressed in the cell membrane and cytoplasm of hepatocytes. Under high magnification, the Kir6.1 protein was observed as punctate immunoreactive products in the cytoplasm of hepatocytes and sinusoidal lining cells (Figure 2B). When the anti-Kir6.1 antibody was omitted or preincubated with antigen peptide, significant immunoreactivity could not be observed (Figure 2D).

Immunoreactivity for Kir6.2 was also weakly distributed in the hepatic lobule (Figure 3A) and in the endothelium of the central vein, the branches of the hepatic artery, and the portal vein in the portal area (Figure 3C). The intensity of immunoreactivity was gradually decreased along the liver plate (Figure 3A and B). Weak immunoreactivity for Kir6.2 was expressed in the cell membrane and cytoplasm of hepatocytes. Under high magnification, the immunoreactive products for Kir6.2 could be observed as rough granules in the cytoplasm of hepatocytes and sinusoidal lining cells (Figure 3B). When the anti-Kir6.2 antibody was omitted or pre-incubated with immunizing antigen peptides, no significant immunoreactivity could be observed (Figure 3D).

Immunoreactivity for SUR1 was quite different from that for Kir6.1 and Kir6.2. Moderate immunoreactivity for SUR1 was distributed in small sinusoidal lining cells with round, ovular, or spindle shapes beside the liver plate (Figure 4A and B), and also in the endothelial cells of the branches of hepatic artery and portal veins at the portal area (Figure 4C). The intensity was not decreased along the liver plate. In hepatocytes, the immunoreactivity for SUR1 was hardly observed in the cytoplasm, and only faintly in the cell membrane (Figure 4B). When the anti-SUR1 antibody was omitted or preincubated with immunizing peptide antigen, the positive immunoreactivity disappeared (Figure 4D).

Immunoreactivity for SUR2A was distributed in the hepatic lobule with moderate intensity around the central vein (Figure 5A), and gradually attenuated along the liver plate. It was mainly localized in irregular- or spindle-shaped cells in the sinusoidal lining (Figure 5B). Weak immunoreactivity for SUR2A was expressed in the cell membrane of hepatocytes. In the portal area, it was weakly expressed in the branches of hepatic arteries and portal veins (Figure 5C).

Immunoreactivity for SUR2B was widely and weakly distributed in the hepatic lobule (Figure 6A). It was mainly localized in small round or oval-shaped cells in the sinusoidal lining. Weak immunoreactivity for SUR2B was expressed in the cell membrane of hepatocytes (Figure 6B). The intensity of immunoreactivity was gradually decreased along the liver plate from the central vein to the distal area (Figure 6A). In the portal area, it was moderately expressed in the branches of the hepatic artery and portal vein, and was especially intense in the bile duct (Figure 6C). When the anti-SUR2A or anti-SUR2B antibodies were omitted or preincubated with each immunizing peptide antigen, the immunoreactivity for SUR2A and/or SUR2B disappeared (Figures 5D and 6D).

Colocalization among K_{ATP} channel subunits and sinusoidal cell marker proteins

To show if pore-forming subunits are co-localized with regulatory subunits in rat liver, immunofluorescence double staining was performed. Positive immunoreactivity of Kir6.1 (Kir6.1$^+$) and Kir6.2 (Kir6.2$^+$) was detected as green fluorescence (Alexa488, Figures 7A, 7D, 8A, 8D, 9A and 9D), and that of SUR1 (SUR1$^+$), SUR2A (SUR2A$^+$) and SUR2B (SUR2B$^+$) was detected as red fluorescence (Alexa594, Figures 7B, 7E, 8B, 8E, 9B, and 9E). In hepatocytes, Kir6.1$^+$ and/or Kir6.2$^+$ were hardly observed to be colocalized with SUR1$^+$, SUR2A$^+$, and/or SUR2B$^+$. Instead, in a small number of round, oval, or spindle-shaped cells located beside the sinusoid, Kir6.1$^+$ and/or Kir6.2$^+$ cells were colocalized with that for SUR1, SUR2A, and/or SUR2B (Figures 7C, 7F, 8C, 8F, 9C, and 9F). When labeled by GFAP, a marker of HSCs, some of the Kir6.1$^+$ and/or Kir6.2$^+$ cells were colocalized with GFAP beside the sinusoid (Figure 10). GFAP$^+$ cells also showed immunoreactivity to SUR1, SUR2A, and SUR2B in the sinusoidal lining (Figure 10). When labeled with CD68, a marker of Kupffer cells, a

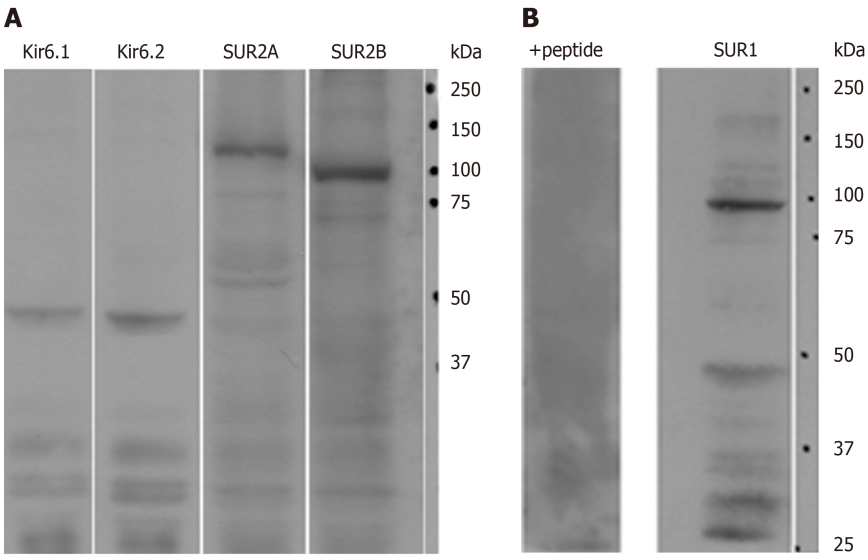


Figure 1 The expression of ATP-sensitive K^+ channel subunits in rat liver extracts. A: Antibodies to Kir6.1, Kir6.2, SUR2A and/or SUR2B showed prominent specific bands, respectively; B: A prominent band around 100 kDa was recognized with our new anti-SUR1 antibody. The band disappeared when blocked by preincubation with the corresponding SUR1 peptide antigen from which the antibody had been raised (+peptide).

few $CD68^+$ cells were colocalized with Kir6.1 and Kir6.2, and more of these small $CD68^+$ cells were showed immunoreactivity to SUR1, SUR2A and SUR2B (Figure 11).

Similarly, when labeled with SE-1, a marker of SECs, a few $SE\text{-}1^+$ cells colocalized with Kir6.1, SUR1, and SUR2B. On the other hand, a number of $SE\text{-}1^+$ cells colocalized with Kir6.2 and SUR2A, respectively (Figure 12).

Primary HSCs were labeled with GFAP antibody. $GFAP^+$ cells also showed positive immunoreactivity for Kir6.1 and/or Kir6.2 (Figure 13). This result indicated that the pore-forming subunits of the K_{ATP} channel were expressed in HSCs. In these HSCs, immunoreactivity for Kir6.1 and Kir6.2 were located in the peripheral cytoplasm, and that for GFAP was located in the perinuclear region. Colocalization of SURs and GFAP in primary HSCs has not been observed.

Primary Kupffer cells showed immunoreactivity for CD68, which confirmed them to be Kupffer cells. When incubating these cells with antibodies of K_{ATP} channel subunits, the $CD68^+$ cells showed immunopositive reactivity for Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B (Figure 14).

Furthermore, when primary Kupffer cells were incubated with multiple paired combined antibodies of pore-forming subunits and regulatory subunits, they showed immunoreactivity for Kir6.1/SUR1, Kir6.1/SUR2A, Kir6.1/SUR2B, Kir6.2/SUR1, Kir6.2/SUR2A, and Kir6.2/SUR2B (Figure 15). The immunoreactive products for K_{ATP} channel subunits exhibited a punctate or granular appearance.

DISCUSSION

In this study, a new polyclonal anti-SUR1 antibody was generated and applied to investigate the exhibition and localization of SUR1 in rat liver. Its specificity was assessed by preabsorption with an immunizing peptide antigen in western blot analysis (Figure 1B). The most significant band at 100 kDa disappeared in the presence of the immunizing antigen, showing the high specificity of the antiserum. The specificity of the antisera for SUR2A and SUR2B was demonstrated elsewhere[35]. The expression of five kinds of K_{ATP} channel subunits in rat liver extract was revealed by western blot analysis with the antibodies, including this new anti-SUR1, and every antibody revealed their corresponding band in the liver extract, respectively (Figure 1). This means that the rat liver contains five kinds of K_{ATP} channel subunits: Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B.

In liver sections, immunoreactivity for Kir6.1, Kir6.2, SUR2A and SUR2B was weakly to moderately expressed around the central vein and gradually decreased along the liver plate (Figures 2, 3, 5, and 6), but the immunoreactivity for SUR1 showed no such tendency (Figure 4). The Kir6.1 and Kir6.2 pore-forming subunits of the K_{ATP} channel were weakly expressed in hepatocytes and sinusoidal lining cells, such as HSCs, Kupffer cells, and SECs. Under high magnification, the Kir6.1 protein

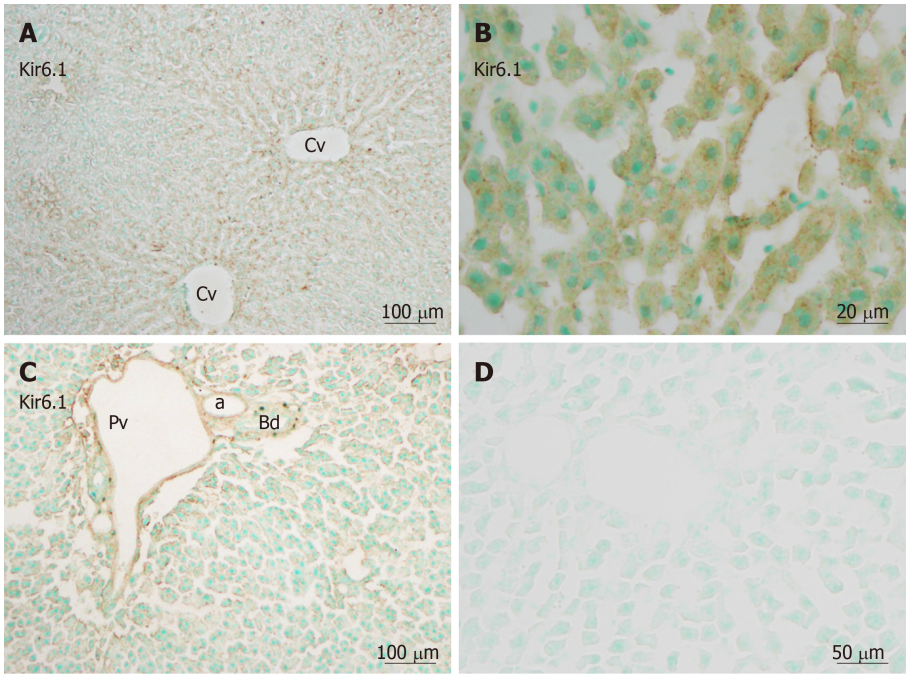


Figure 2 Kir6.1 immunoreactivity in liver sections. Kir6.1 was expressed in hepatocytes and sinusoidal cells of the liver. A: Staining was intense in the area around the central vein; B: Showing punctate reactivity around the nucleus in hepatocytes cells and sinusoidal cells when viewing with high magnification; C: Weaker immunoreactivity was evident in the triangular areas of the vein and artery, but no reactivity was evident in the bile duct; D: A negative control section devoid of staining. a: Artery; Bd: Bile duct, Cv: Central vein; Pv: Portal vein. Bars: 100 μm (A and C), 20 μm (B), 50 μm (D).

was detected as punctate immunoreactive products (Figure 2B) and the Kir6.2 protein was detected as granular immunoreactive products, indicating that Kir6.1 was located in the mitochondria and Kir6.2 in the endoplasmic reticulum, as shown in previous studies on rat brain[8,9]. The regulatory subunits SUR1, SUR2A, and SUR2B were mainly located in small cells, and exhibited staining in sinusoidal cells and weak staining in the cell membrane of hepatocytes.

The K_{ATP} channels play important roles in hyperglycemia, hypoglycemia, ischemia and hypoxia[16,36]. Kir6.1 mRNA and protein are upregulated during myocardial infarction[17,37,38]. When the liver is in ischemia followed by reperfusion, hepatocytes around the central veins were more markedly degenerated than those in the peripheral zone of the liver lobule[38]. During liver ischemia, the different degenerated zonal areas are accordant with the liver blood supply being made up of different gradients of oxygen, hormones, and metabolites, as well as zonal differences form periportal to perivenous[39,40]. The similar zonal differences in immunoreactivity for Kir6.1, Kir6.2, SUR2A, and SUR2B might indicate that these subunits of K_{ATP} channels have some relationship with the protection of hepatocytes, though colocalization of these subunits was hardly observed in the sections of normal rat liver. K_{ATP} channels are important in liver regeneration for keeping a higher ATP content in liver tissue during partial hepatectomy[15] since, clinically, hepatocyte regeneration is a key point for partial liver transplantation.

It is well known that, in addition to parenchymal cells, the liver consists of three other kinds of cells, *i.e*. HSCs, Kupffer cells, and SECs[41]. HSCs are located between hepatocytes and SECs[31]. They store large amounts of vitamin A and produce extracellular matrix for liver fibrogenesis[31,41]. The pore-forming subunits (Kir6.1 and Kir6.2) were clearly colocalized in HSCs with GFAP both in liver sections and primary cells (Figures 10 and 13). Although the regulatory subunits (SURs) were not yet observed in primary HSCs, they were colocalized in HSCs with GFAP in liver sections (Figure 11). These results might indicate that the K_{ATP} channels have some relation to HSC matrix secretion and fibrogenesis.

In addition to the metabolism of glucose, lipids, hormones, and various substances, the liver also plays important roles in the immune response to eliminate pathogens, such as microorganisms, endotoxins, portal antigens, and tumor cells, and Kupffer cells are responsible for these crucial functions of host defense[42]. There are several subsets of Kupffer cells categorized by the cytokines they produce, such as $CD11b^+$, $CD68^+$, $CD11b^-$ and/or $CD68^-$, CD32, and so on[40,43]. Kupffer cells can also be divided

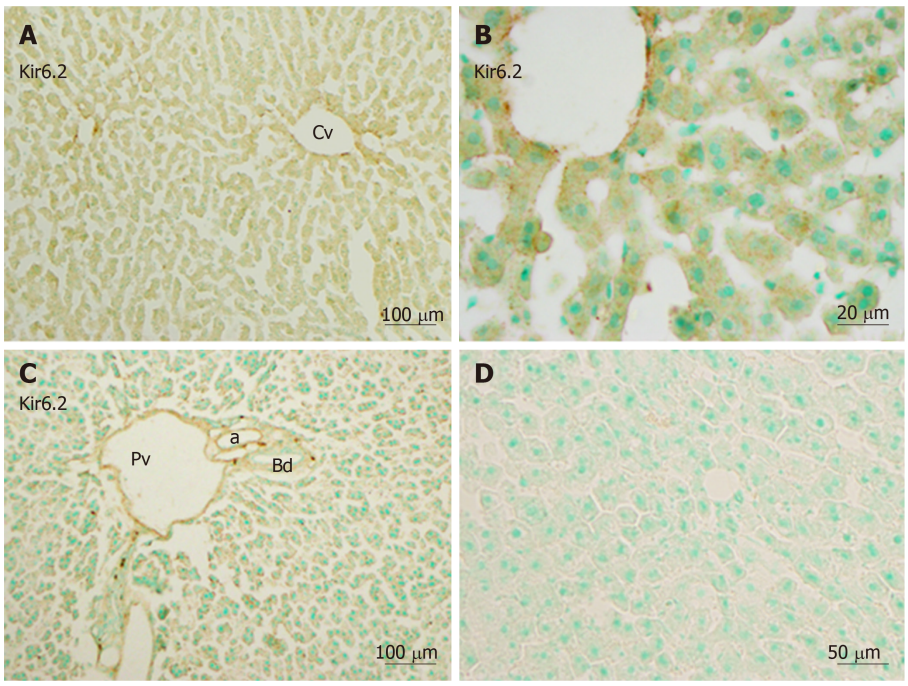


Figure 3 Immunoreactivity with Kir6.2 was expressed in hepatocytes and sinusoidal cells of the liver. A and B: Mediated immunoreactivity was seen in the area around the central vein; A and C: Mediated immunoreactivity was weaker in the border areas; D: A negative control section devoid of staining (no more than background staining was observed). a: Artery; Bd: Bile duct, Cv: Central vein; Pv: Portal vein. Bars: 100 μm (A and C), 20 μm (B), 50 μm (D).

into two groups: bone marrow-derived macrophages and sessile hepatic macrophages. These different groups of Kupffer cells play different roles in the liver. For instance, when mice were given food with high-fat or high-cholesterol, $CD68^+$ Kupffer cells decreased while $CD11b^+$ Kupffer cells increased in number[40]. K_{ATP} channels exhibit different physiological and pharmacological functions depending on their subunit composition[26]. For example, Kir6.2 with SUR1 is strongly sensitive to the K_{ATP} channel activator diazoxide, but not to other activators such as pinacidil[25], while Kir6.2 with SUR2A is strongly sensitive to pinacidil and cromakalin but only weakly to diazoxide[26]. The diversity of subunit composition of K_{ATP} channels in Kupffer cells might correspond to different types of Kupffer cells or to their various functional stages.

In SECs, SE-1 immunopositive cells were colocalized in different numbers with the five kinds of K_{ATP} channel subunits, respectively (Figure 12). It is well known that SECs are highly specialized endothelial cells (with different classifying types) located between blood, hepatocytes and HSCs[31]. In the physiological state, SECs have a low proliferation rate. They maintain HSC quiescence, thus inhibiting intrahepatic vasoconstriction and fibrosis development under normal conditions. They are also differentially renewable under physiological and pathological conditions[44]. They are also involved in liver regeneration following acute liver injury and partial hepatectomy[45]. They play a key role in promoting liver regeneration by orchestrating the harmonious regeneration of the different cell types and renew themselves with asynchronous liver regeneration[44]. In the present study, SECs showed different combinations of Kir6.x and SURs, which indicates that they have multiple kinds of K_{ATP} channels corresponding with their multiple functions under physiological and pathological conditions.

The present study is, to our knowledge, the first report that HSCs, Kupffer cells, and SECs contain K_{ATP} channel subunits. Further investigation is needed to elucidate their functions in detail in liver metabolism, immunity, and fibrogenesis during liver injury.

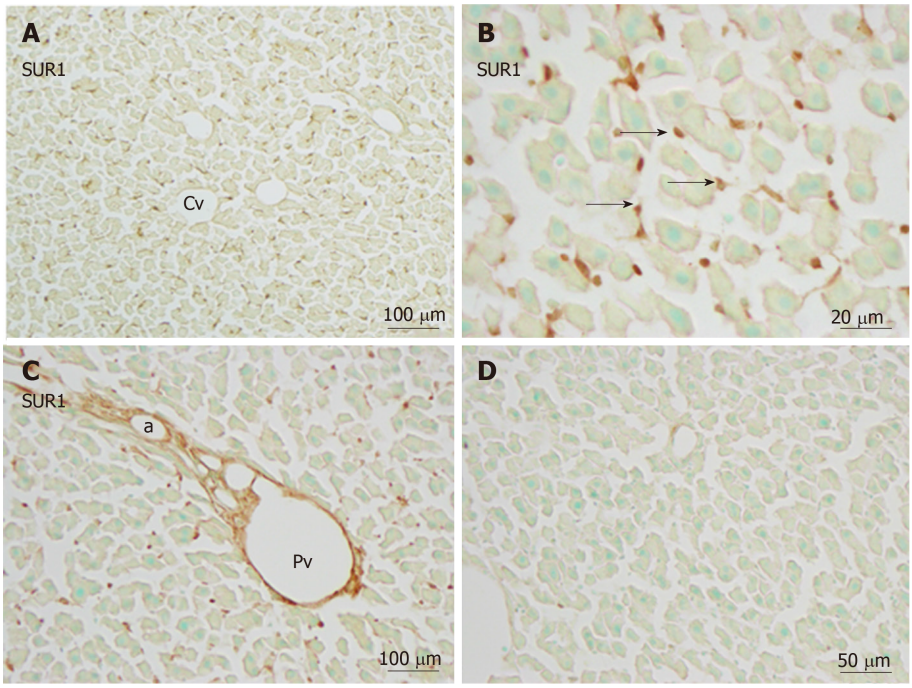


Figure 4 Immunoreactivity with SUR1 was mainly evident in sinusoidal cells (arrows) and weaker in the cell membranes of hepatocytes. A and B: There was no such tendency toward immunoreactivity with SUR1 in the area of the central vein *vs* the border area; C: In the portal area, endothelial cells of the blood vessels showed intense immunoreactivity with SUR1; D: Absorption negative control section devoid of staining (no more than background) after incubation with cognate antigen peptide. a: Artery, Cv: Central vein; Pv: Portal vein. Bars: 100 μm (A and C), 20 μm (B), 50 μm (D).

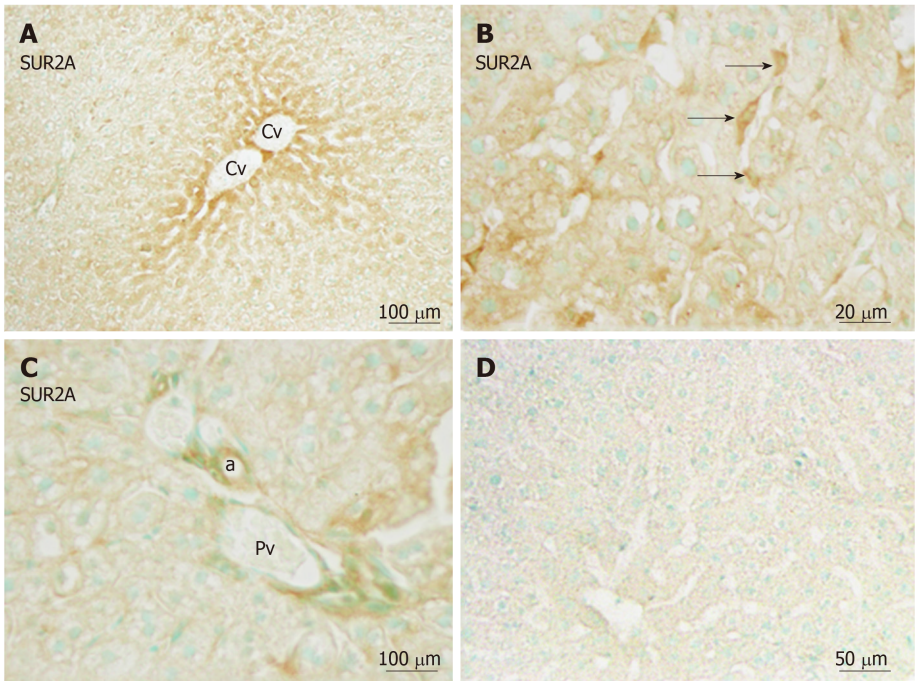


Figure 5 Immunoreactivity with SUR2A was evident in hepatocytes and sinusoidal cells (arrows) of the liver. A: Immunoreactivity with SUR2A was stronger in the area of the central vein (Cv) *vs* the border areas; B: Weaker immunoreactivity in the cell membrane and moderate immunoreactivity in the sinusoidal cells were observed (arrows); C: Weaker immunoreactivity with SUR2A was also observed in the endothelial cells of the blood vessels; D: Absorption negative control section devoid of staining (no more than background was observed). a: Artery. Cv: Central vein; Pv: Portal vein. Bars: 100 μm (A and C), 20 μm (B), 50 μm (D).

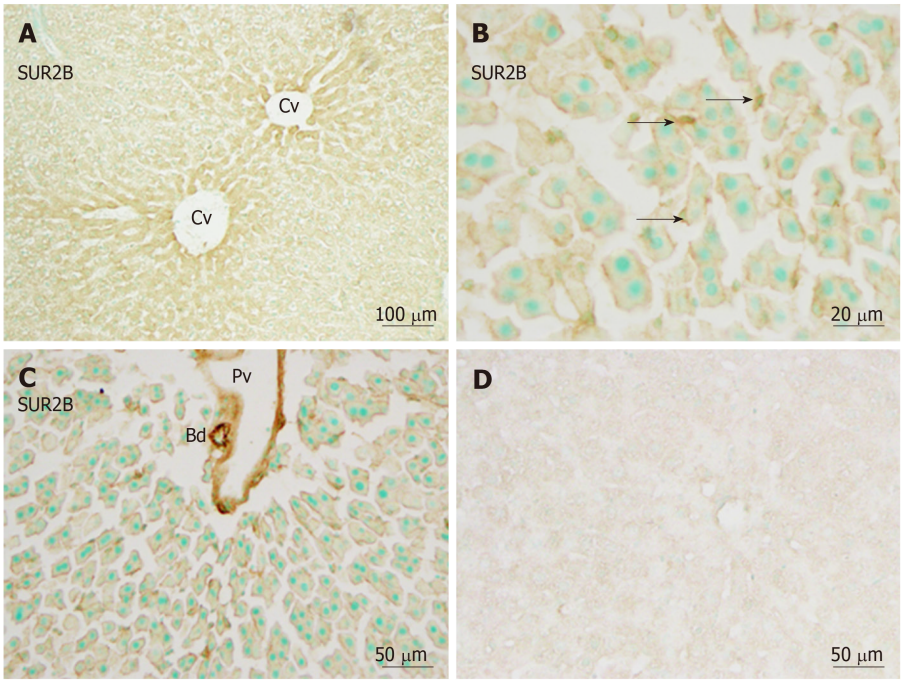


Figure 6 Immunoreactivity with SUR2B. A: Immunoreactivity with SUR2B was stronger in the area of the central vein (Cv) *vs* the border areas; B: Immunoreactivity with SUR2B was expressed in hepatocytes and sinusoidal cells (arrows) of the liver. Weaker immunoreactivity in the cell membrane and medium immunoreactivity in sinusoidal cells was observed (arrows); C: Immunoreactivity with SUR2B was also observed in the endothelial cells of blood vessels and bile ducts; D: Absorption negative control section devoid of staining (no more than background was observed). Bd: Bile duct; Cv: Central vein; Pv: Portal vein. Bars: 100 μm (A), 20 μm (B), 50 μm (C and D).

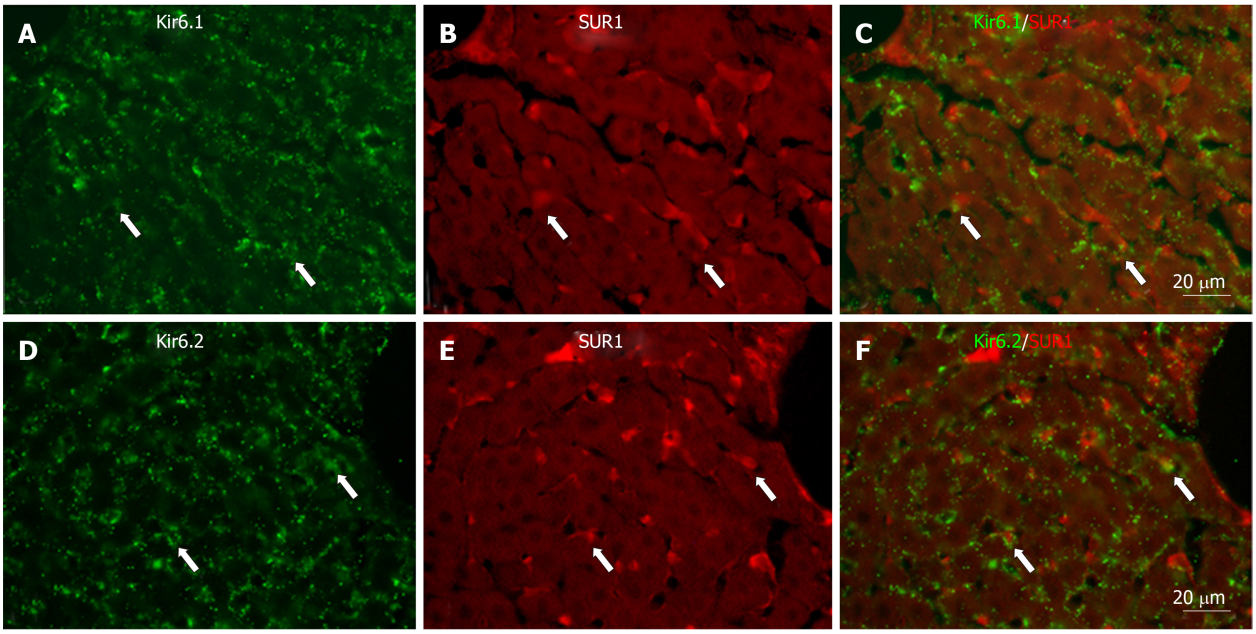


Figure 7 Immunofluorescence double staining for Kir6.1 and/or Kir6.2 with SUR1 in liver sections. A and D: Kir6.1 and Kir6.2 were widely localized in hepatocyte and sinusoidal cells; B and E: SUR1 was mainly localized in sinusoidal cells; C: There were few sinusoidal cells that had colocalized Kir6.1 and SUR1 (arrows); F: There were few sinusoidal cells that had colocalized Kir6.2 and SUR1 (arrows). Bars: 20 μm.

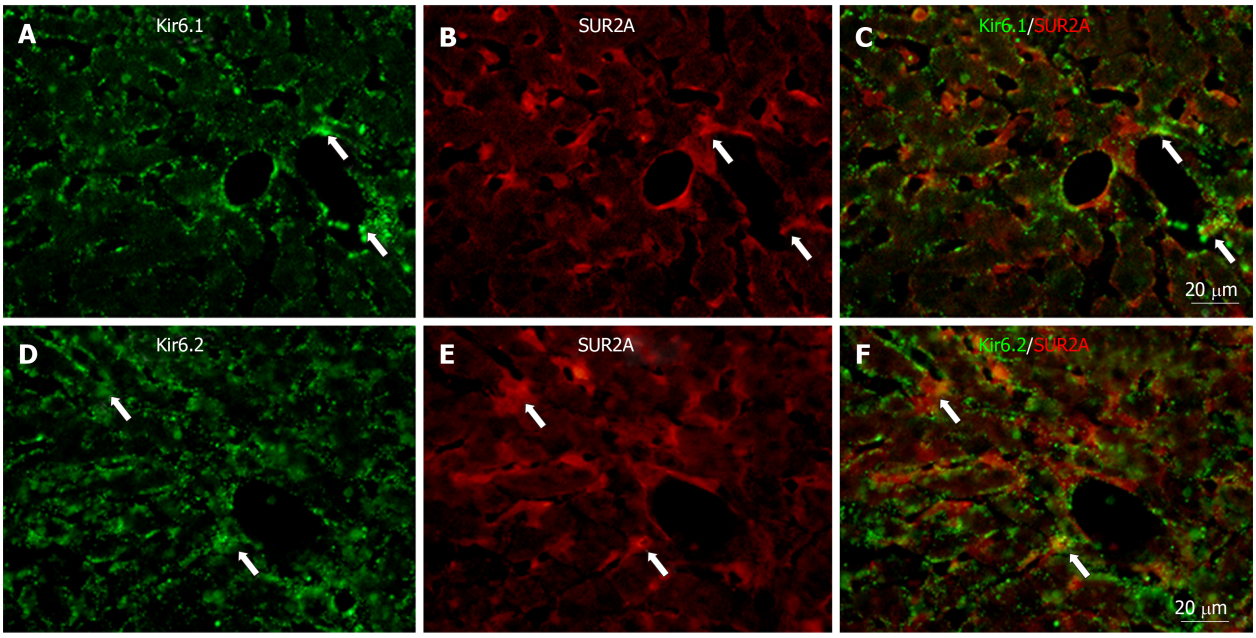


Figure 8 Immunofluorescence double staining for Kir6.1 and/or Kir6.2 with SUR2A in liver sections. A and D: Kir6.1 and Kir6.2 were widely localized in hepatocyte and sinusoidal cells; B and E: SUR2A was mainly localized in sinusoidal cells; C: There were few sinusoid cells with colocalized Kir6.1 and SUR2A (arrows); F: There were few sinusoidal cells with colocalized Kir6.2 and SUR2A (arrows). Bars: 20 μm.

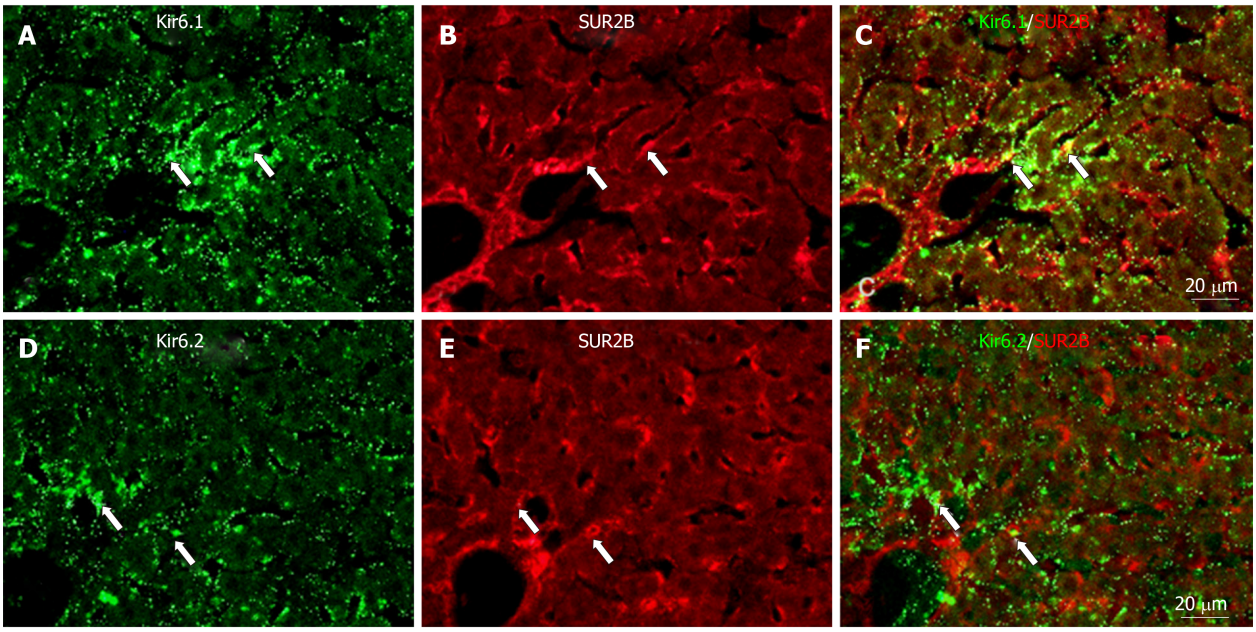


Figure 9 Immunofluorescence double staining for Kir6.1 and/or Kir6.2 with SUR2B in liver sections. A and D: Kir6.1 and Kir6.2 were widely localized in hepatocyte and sinusoidal cells; B and E: SUR2B staining was mainly present in sinusoidal cells; C: There were few sinusoidal cells with colocalized Kir6.1 and SUR2B (arrows); F: There were few sinusoid cells with colocalized Kir6.2 and SUR2B (arrows). Bars: 20 μm.

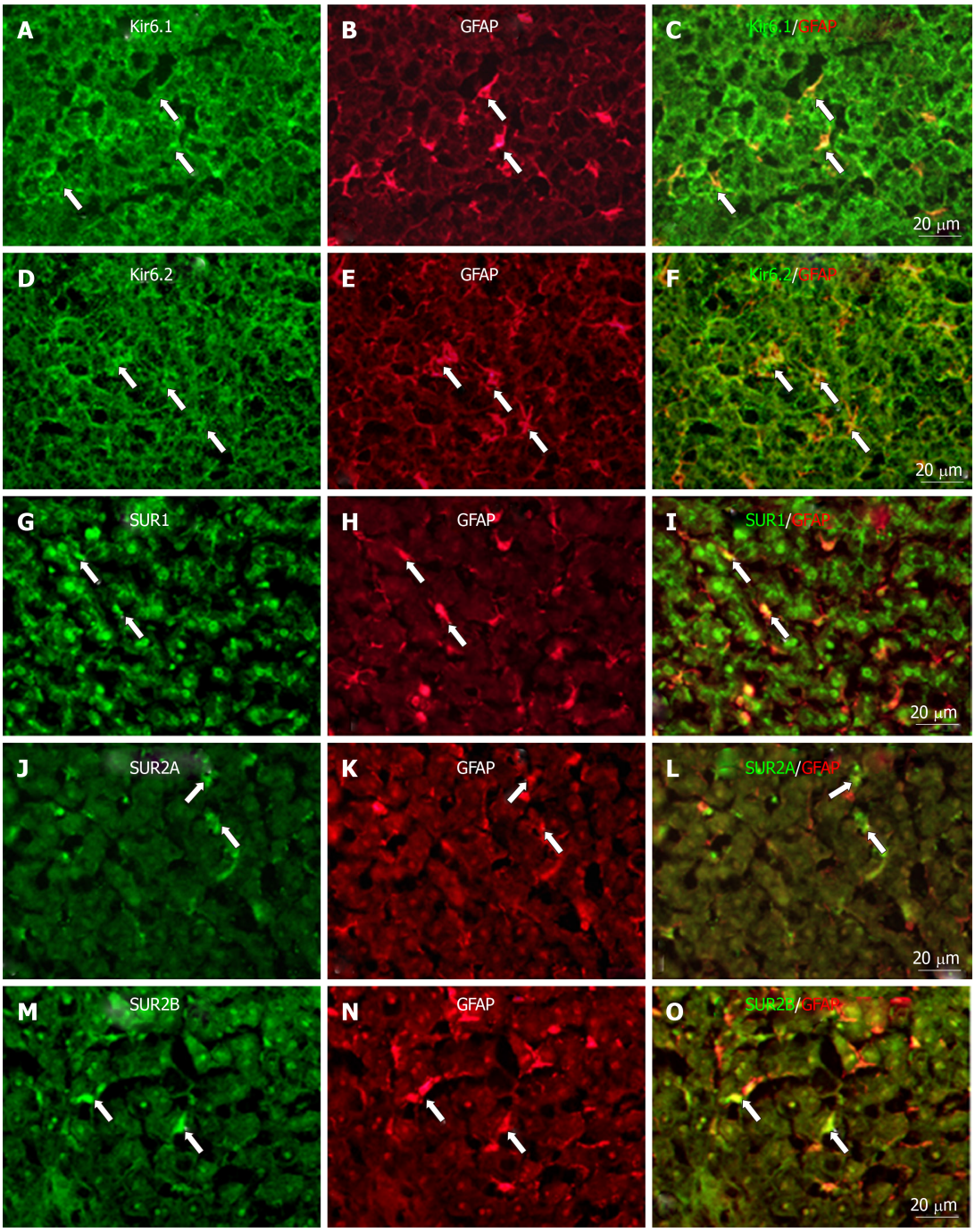


Figure 10 Immunofluorescence double staining. Immunofluorescence double staining for Kir6.1, Kir6.2, SUR1, SUR2A, and/or SUR2B (A, D, G, J, and M) and GFAP [B, E, H, K, and N, marker of hepatic stellate cells (HSCs)] in no-fixed liver sections; Most of the $GFAP^+$ cells (HSCs) colocalized with Kir6.1 (C, arrows) and/or Kir6.2 (F, arrows); There were few $GFAP^+$ cells (HSCs) colocalized with SUR1 (i, arrows), SUR2A (L, arrows), and/or SUR2B (O, arrows). Bars: 20 μm.

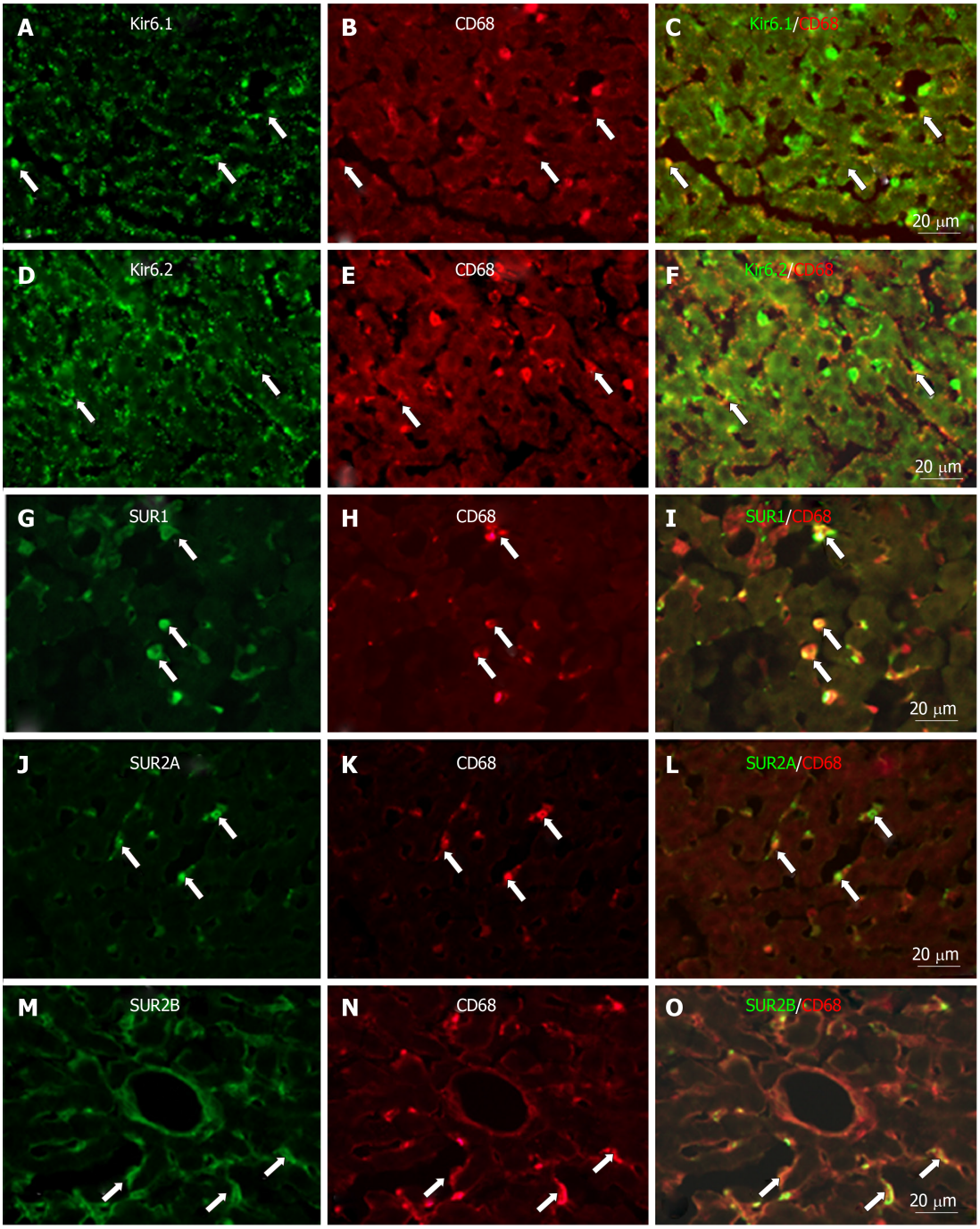


Figure 11 Immunofluorescence double staining for ATP-sensitive K^+ channel subunits Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B with CD68 in liver sections. Immunofluorescence double staining for ATP-sensitive K^+ channel subunits Kir6.1 (A), Kir6.2 (D), SUR1 (G), SUR2A (J) and SUR2B (M) with CD68 (B, E, H, K, and N, marker of Kupffer cells) in liver sections; Some $CD68^+$ cells (Kupffer cells) colocalized with Kir6.1 (C, arrows) and/or Kir6.2 (F, arrows), but most colocalized with SUR1 (I, arrows), SUR2A (L, arrows) and SUR2B (O, arrows). Bars: 20 μm.

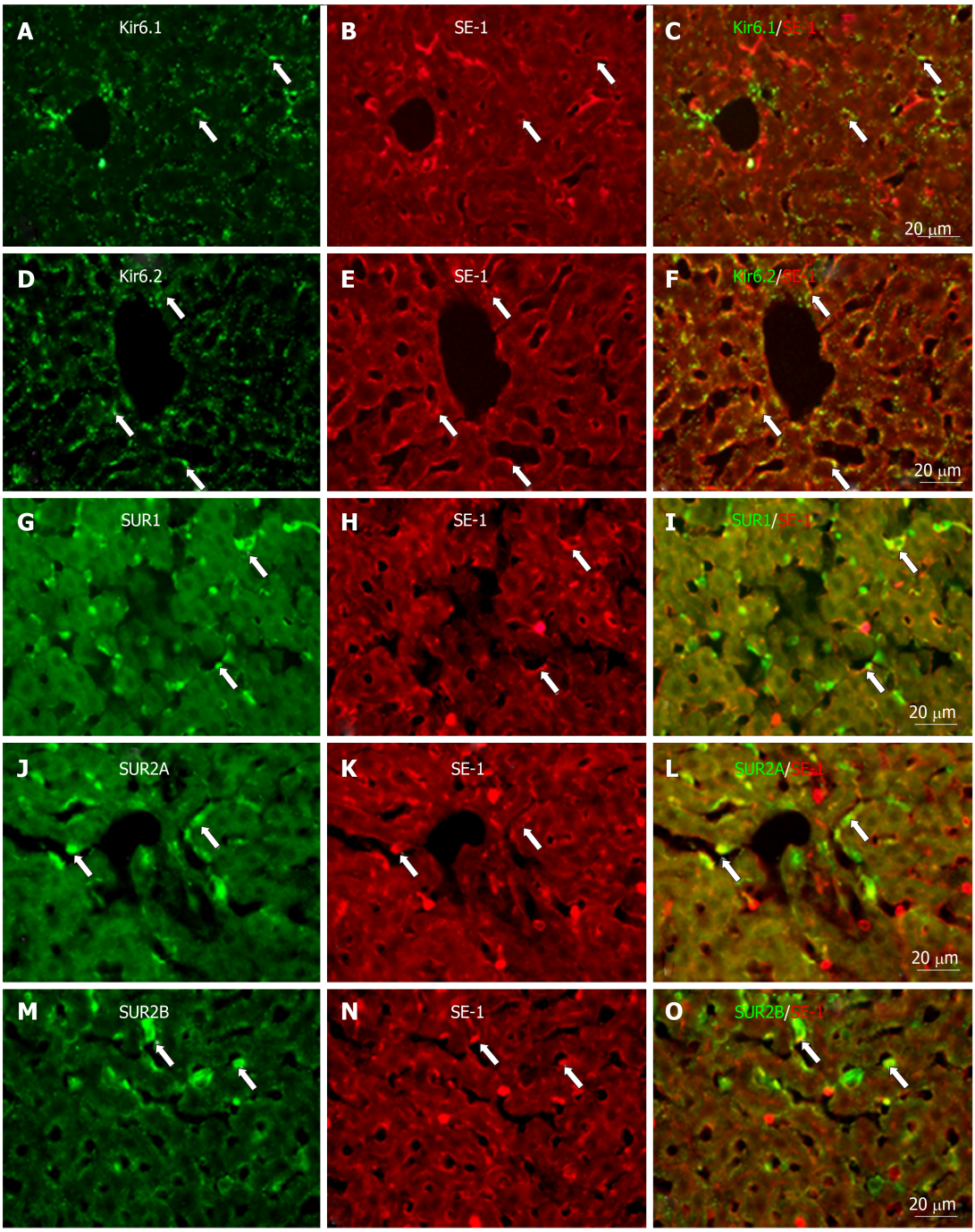


Figure 12 Immunofluorescence double staining for ATP-sensitive K^+ channel subunits Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B with SE-1. Immunofluorescence double staining for ATP-sensitive K^+ channel subunits Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B (A, D, G, J, and M) with SE-1 [marker of sinusoidal endothelial cells (SECs)]; In the liver sections, SECs showed immunopositive reactivity to SE-1 (B, E, H, K, and N); A few $SE\text{-}1^+$ cells colocalized with Kir6.1 (C, arrows), but a number of them colocalized with Kir6.2 (F, arrows); As for SURs, some $SE\text{-}1^+$ cells colocalized with SUR1 (I, arrows) and/or SUR2B (O, arrows); Most $SE\text{-}1^+$ cells colocalized with SUR2A (L, arrows). Bars: 20 μm.

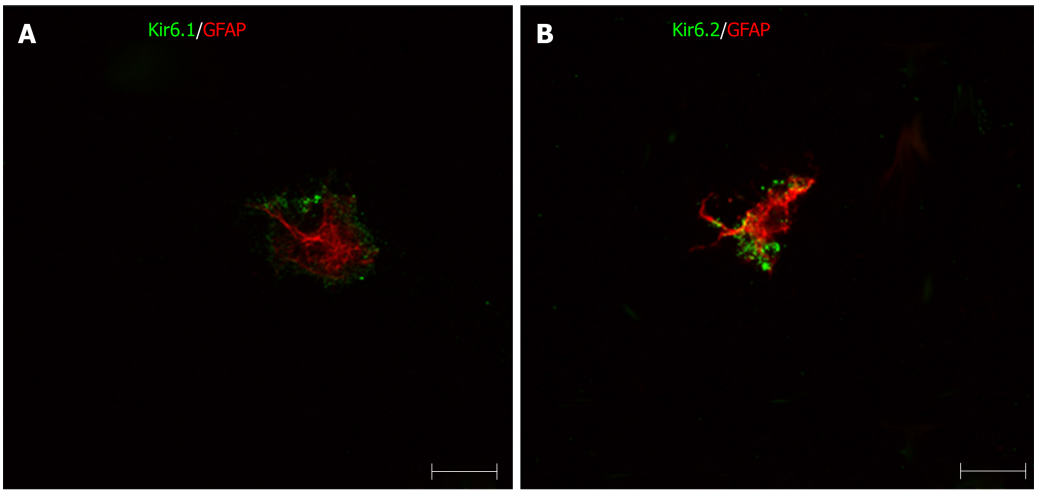


Figure 13 Immunofluorescence double staining for pore-forming subunits in primary cultured hepatic stellate cells. The primary hepatic stellate cells were marked by GFAP (red), they showed immunoreactivity to Kir6.1 and Kir6.2 (green). Bars: 50 μm.

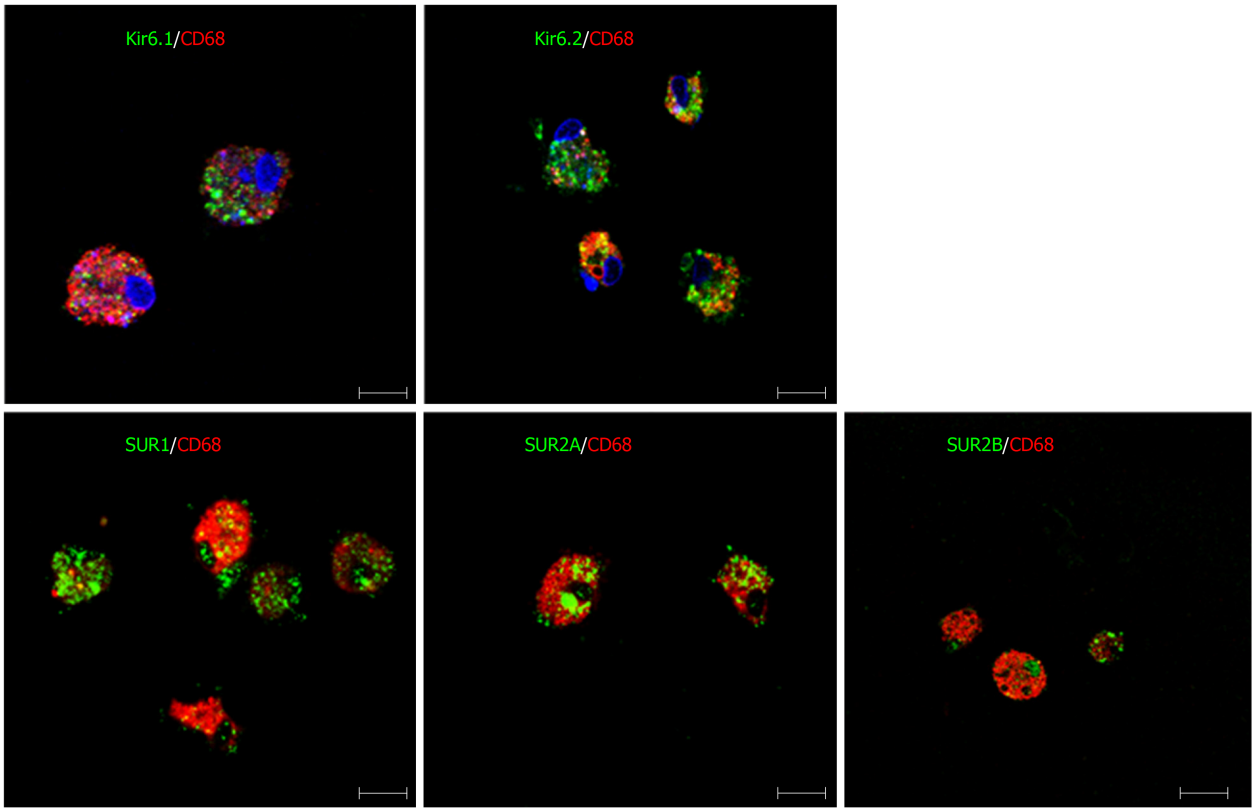


Figure 14 Immunofluorescence double staining for ATP-sensitive K^+ channel subunits in primary cultured Kupffer cells. Primary Kupffer cells were labeled with CD68 (red). These $CD68^+$ Kupffer cells also showed immunoreactivity to five kinds of ATP-sensitive K^+ channel subunits: Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B (green). Bars: 50 μm.

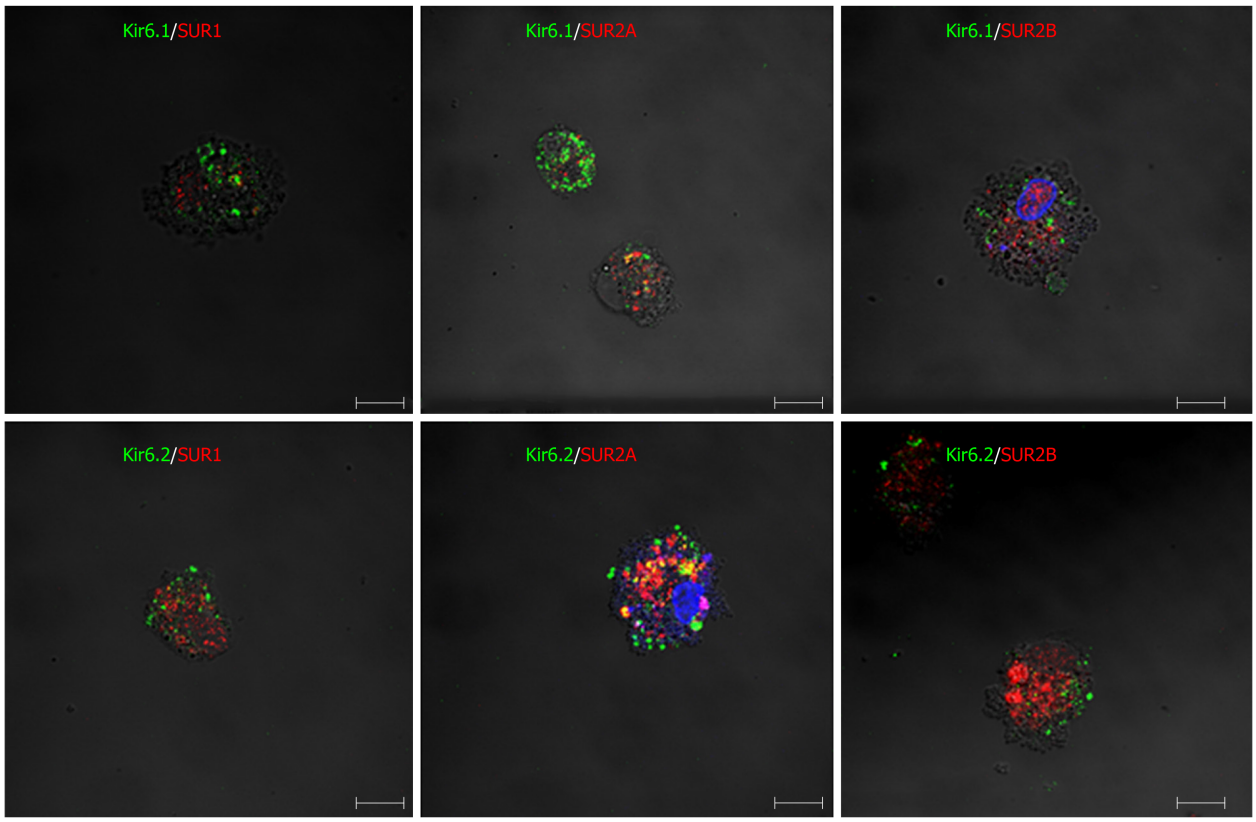


Figure 15 Immunofluorescence double staining for combinations of pore-forming subunits and regulatory subunits in primary cultured Kupffer cells. In primary Kupffer cells, the pore-forming subunits Kir6.1 and Kir6.2 are labeled in green, and the regulatory subunits SUR1, SUR2A and SUR2B are labeled in red. The combinations of Kir6.1/SUR1, Kir6.1/SUR2A, Kir6.1/SUR2B, Kir6.2/SUR1, Kir6.2/SUR2A and Kir6.2/SUR2B are all exhibited in the Kupffer cells. Bars: 50 μm.

ARTICLE HIGHLIGHTS

Research background

ATP-sensitive K^+ (K_{ATP}) channels coupling nutrient metabolism to membrane potential are sensitive to intracellular ATP concentrations and ATP/ADP ratios. K_{ATP} channels are hetero-octameric in composition, formed by four pore-forming subunits including Kir6.1 and Kir6.2, which belong to the inward rectifying potassium channel family, and four regulatory subunits, SUR1, SUR2A and SUR2B, which belong to members of the ATP-binding cassette protein family. K_{ATP} channels were considered to be involved in cellular functions, such as pancreatic β cell secretion, muscle and neuronal excitability, and so on.

Research motivation

How K_{ATP} channels work in liver hepatocytes and sinusoidal cells is important to help understand liver function under normal and injury conditions. The first step is to characterize the localization and distribution of K_{ATP} channel subunits in different cells of the liver. This research focused on the localization and distribution of K_{ATP} channel subunits in hepatocytes and sinusoidal cells of rat liver. This research will provide the basic data to guide further research in medicine exploitation and clinical treatment for related liver diseases.

Research objectives

To investigate the expression of K_{ATP} channel subunits in rat liver and their localization in different cells of the liver.

Research methods

In order to obtain basic knowledge of K_{ATP} channel subunits in rat liver distribution and localization, extracted liver protein was used for immunoblot analysis, cryosections of liver were used for immunohistochemistry, and primary cultured hepatic stellate cells (HSCs) and Kupffer cells were used for immunocytochemistry.

Research results

In the present study, five types of K_{ATP} channel subunits, including Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B, were expressed in rat liver as revealed by immunoblot analysis and immunohistochemistry. Double immunofluorescence staining of cryosections, primary cultured HSCs and Kupffer cells showed that not only hepatocytes, but also HSCs, Kupffer cells and

sinusoidal endothelial cells (SECs) contain K_{ATP} channel subunits.

Research conclusions

The present study revealed that K_{ATP} channel subunits are expressed in rat liver, which is the first report that HSCs, Kupffer cells, and SECs contain K_{ATP} channel subunits formed by Kir6.1/SUR1, Kir6.1/SUR2A, Kir6.1/SUR2B, Kir6.2/SUR1, Kir6.2/SUR2A, and Kir6.2/SUR2B. Different combinations of K_{ATP} channels have different physiological and pharmacological characteristics. Thus, this research will supply basic experimental data to guide further exploration of liver function under normal and injury conditions.

Research perspectives

In the present study, all experiments were performed under normal conditions. Thus, further study is needed to elucidate changes in K_{ATP} channel subunit composition under different metabolic states, including ischemia and hypoxia in vitro and/or in vivo with the use of K_{ATP} channel openers and blockers, such as diazoxide, pinacidil, glibenclamide, and tolbutamide.

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