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Applied genomic in cerebrovascular disease

Genómica funcional en la enfermedad vascular cerebral

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Abstract

Cerebrovascular disease involve the alterations caused by pathology process of the sanguineous vessels, affecting one or many brain areas. Cerebrovascular disease is also known like stroke or ictus; it is the third cause of death around the world and is the neurologic pathology with the most prevalence rate. Cerebrovascular disease induces several changes in genetic expression inside the neurovascular unit (glia cells, neurons and ependymal cells); principally, changes in the oxidative stress and calcium inflow into the cells, this could start cellular death and tissue destruction, causing an irreversible injury in brain, losing several functions. The injury causes the activation of signaling pathways to respond to the stress, where many molecules such as proteins and mRNA are involved to act as intermediaries to activate or deactivate stress mechanisms; these molecules are able to transmit extracellular signals into the nucleus activating early gene expression like proto-oncogenes and several transcription factors to repair the cerebral injury. It is important to know the relation of the changes in genetic expression and proteins to avoid the development of injury and to activate the brain recovery. This knowledge let us diagnose the injury rate and propose therapeutic mechanisms to reduce or avoid the adverse effects on time, before the cellular death start.

Key words: Cellular adaptation, cerebrovascular disease, genetic expression, experimental models.

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Resumen

Las enfermedades cerebrovasculares implican las alteraciones causadas por el proceso patológico de los vasos sanguíneos, que afectan a una o varias áreas del cerebro. La enfermedad cerebrovascular también se conoce como ictus o ictus; Es la tercera causa de muerte en todo el mundo y es la patología neurológica con mayor tasa de prevalencia. La enfermedad cerebrovascular induce varios cambios en la expresión genética dentro de la unidad neurovascular (células gliales, neuronas y células ependimales); Principalmente, los cambios en el estrés oxidativo y la entrada de calcio en las células, podrían iniciar la muerte celular y la destrucción del tejido, causando una lesión irreversible en el cerebro, perdiendo varias funciones. La lesión hace que la activación de las vías de señalización responda al estrés, donde muchas moléculas, como las proteínas y el ARNm, actúan como intermediarios para activar o desactivar los mecanismos de estrés; estas moléculas son capaces de transmitir señales extracelulares en el núcleo activando la expresión génica temprana como protooncogenes y varios factores de transcripción para reparar la lesión cerebral. Es importante conocer la relación de los cambios en la expresión genética y las proteínas para evitar el desarrollo de lesiones y activar la recuperación del cerebro. Este conocimiento nos permite diagnosticar la tasa de lesiones y proponer mecanismos terapéuticos para reducir o evitar los efectos adversos a tiempo, antes de que comience la muerte celular.

Palabras clave: adaptación celular, enfermedad cerebrovascular, expresión genética, modelos experimentales..

CEREBROVASCULAR DISEASE

The right cell and organs function depends on a constant sanguineous flow to supply oxygen and nutrients to keep a right hydric balance and delete the toxic metabolic residues to the organism (1,2). The altered vascular permeability could produce injury even with intact sanguineous flow (3).

Vascular cerebral disease (CVD) is any brain alteration produced by a pathological process in sanguineous vessels (4). The Latin term "Ictus" means "stroke", and involves all these pathologies, specifically when the symptoms are sudden and acute (5). According to their nature, CVD could be a deficiency in the flow caused by thrombosis or embolism (ischemia), also known commonly as cerebrovascular ischemic disease as well as hemorrhagic cerebral disease, caused in the parenchyma or inside the cerebral ventricles (cerebral hemorrhagic), or in the subarachnoid spice (subarachnoid hemorrhage), in a proportion of 85% and 15% respectively (figure 1) (3, 4, 6).

The CVD is the third cause of mortality around the world (7) and recently in Mexico it reached the same position (8), just after cancer and heart disease (figure 2). In addition, is the most prevalent neurological pathology and one of the main causes of disability in the world (9, 10).

Cerebrovascular ischemic disease: cerebral ischemia

Cerebrovascular ischemic disease causes the decrease of glucose and oxygen in brain tissue. This could be in the whole brain, also known as global cerebral ischemia or in localized zones known as focal cerebral ischemia or multifocal if there are many zones affected. Specifically, in focal cerebral ischemia, if the ischemic process arises for 2-15 minutes it is a transitory ischemic attack or if the cellular death is present is a cerebral infarction where the sanguineous flow was restored during 24 hours after the ischemic event. The recovery, of sanguineous reperfusion after the

ischemic attack is denominated sanguineous reperfusion, and sometimes, in many cases the damage is increased, since the cells have changed the mechanism for the adaptation against the hypoxia and the abrupt change in oxygen level is not supported by cells, increasing the production of reactive oxygen species and the oxidative stress originate in the ischemic zone. Then, the sanguineous flow recovery with respect to time, is essential for an adequate recuperation (5, 11).

When the sanguineous flow stops the regulated balance among the components of the neurovascular unit, the excitotoxity emerges in the cells , along with the oxidative stress, inflammation, intracellular signaling and neuronal death (12, 13). These events cause changes directly and indirectly in gene expression and in most cases as consequence, changes in protein levels (14, 15).

GENETICS AND CEREBRAL ISCHEMIA

CVD induces changes in genetic expression related to repair and cell recovery (16). With ischemia, the energetic metabolism fails and the mRNA levels diminish severely in the ischemic zone (14, 17). On the other side, the early expression genes are activated and support the neuronal survival like c-fos, c-jun, jun-B, jun-D, krox-20, zif268 y homer 1a (18-22), that cause the expression of other genes through the protein-1 and the cAMP response element mainly, subsequently the heat shock proteins 70 (HSP70) are expressed and many cytokines as tumoral necrosis factor alpha (TNFα), interleukin 1β (IL1β), interleukin 6 (IL6) and the quimiotactic peptide 1 of the monocyte (12, 23, 24). These cytokines induce the adhesion molecules expression as intercellular adhesion molecule 1, adhesion molecule 1 endothelial of leukocyte and the P-secretin in the cerebral vascularization, originating the inflammatory response. Subsequently, the genetic expression, changes in several compartments like phosphodiesterase 4D (PDE4D) increasing in mononuclear cells from peripheric blood (25). The cytokines activate the gene expression related with the inflammatory response like the inducible nitric oxide synthase and the cyclooxygenase 2. With respect to nitric oxide, it has been related with neuroprotection mechanisms preconditioned by a possible neurogenesis factor, Idunna (goddess of protection and ever youth), named like this for the protector effects caused by its expression (21).

After cerebral ischemia genes related with the apoptosis like p53 and bax (19), proapoptotic factors like the tumoral necrosis Fas and Apo-2L, the death receptor TR3, the nuclear factor kB (NFkB) and genes linked to apoptosis 2 (ALG2) and Pip92 (26, 27) are activate besides these condition, it has been detected the antiapoptotic factors like Bcl2 protein, the tumoral growth factor β1 (TGF-β1), transformation and growth factor alpha (TGFα), the erythropoietin and the growth factor related to insulin. The neuron capacity to express each one of these factors is related with its specific vulnerability to the ischemia and to the apoptotic or necrotic death (26).

EXPERIMENTAL MODELS TO CEREBROVASCULAR DISEASE

To study the CVD, *in vitro* and *in vivo* experimental models have been used, to replicate the physiopathological process after ischemia in the human brain. These models have generated information about molecular mechanisms triggered due to cerebrovascular disease; in addition, it has been possible to know the normal organization

of several neurotransmitter systems, neurochemistry and neuropharmacology, because the biochemical effects in cells and tissues due to ischemia are replicated, constituting an advantage to study the CVD (28, 29). These experimental models allow the application of global study methods, where it is possible to study simultaneously several and different expressed molecules. These systems include gene differential analysis and RNA microarray assay to study changes in genetic expression (30) and the several effector proteins they codify by proteomic analysis, based on the identification of the proteins by mass spectrometry analyzing qualitative and quantitative changes in their expression, comparing their behavior like changes in synthesis or alteration in posttranslational modifications in the presence of several experimental ischemic conditions (17, 31).

Several experimental studies have been made in rats and humans where the altered gene expression is analyzed with early and late ischemic brain conditions (32, 35), validating the experimental models *in vitro* and *in vivo* to research the CVD (36, 38), founding therapeutics alternatives to several conditions when the CVD is present; the current treatments are not that all effective and time is a decisive factor in the CVD (14, 36, 39). One of the experimental models to study the CVD is the *post mortem* condition in humans for the precise information to analyze directly the molecular events originated by ischemia, before the total brain tissue damage and necrosis death, representing a great advantage due to the experimental control on the time (40).

Studies realized in our laboratory with ischemia model in rats were analyzed with differential display in three cerebral regions: frontal, hippocampus and occipital zone with 15 minutes of ischemia. Results showed changes in mRNA; in frontal region diminished the catalase gene expression involved in reactive oxygen species protection during ischemia; in the hippocampus increased the mRNA expression of the epithelial glycoprotein 314 (EGP-314) and the ceruplasmin involved in the cellular adhesion and reactive oxygen species protection respectively. The protein L31 gene involved in proliferation process and cellular differentiation, increased in the same zone. Finally, in the occipital region diminished the mRNA expression of the cytochrome c oxidase polypeptide I, that initiates the apoptosis. These changes showed the several mechanisms activated in different cerebral regions involved in the response participating in the damage or cell recovery (41). In addition, we also analyzed changes in protein levels in hippocampus, striatum body and parietal cortex, specifically the actin gamma protein diminished in the three cerebral regions (31), while in in the parietal cortex, phosphatidylethanolamine binding protein 1 diminished and 14-3-3 gamma increased (no published results).

ANALYSIS OF CEREBROVASCULAR DISEASE WITH MICROARRAY TECHNOLOGY

Recently, gene expression massive studies have been made with microarray technology in several experimental models (17, 42-45). Specifically, a study with cerebral cortex cells of mouse cultivated *in vitro* subjected to 24 hours of hypoxia showed 11, 200 genes; 1, 405 changed their expression levels (12.5%), 26 were induced (several involved in the cellular death process due to neuronal vulnerability) and 20 stopped their expression. Other genes found with changes are involved in the endoplasmic reticulum proteins transportation

during ischemia. Also changed genes involved in ubiquitination pathways; for degradation or activation of signaling intracellular pathways. Finally, several genes changed their expression involved in neuroprotection and tissue damage (indirectly) expressed in non-neuronal hypoxic systems (46).

Several microarray studies have been made with different hypoxic and ischemic conditions in neonatal mice (2, 8, 24).

Another microarray study was made with distinct hypoxia and ischemia periods of time in neonatal mice (2, 8, 24). The differential expression of 343 genes were observed in cortex, hippocampus, thalamus and striatum; 283 genes increased and 60 diminished their expression. The upregulated genes encode translational factors and intermediary metabolism, while genes with down regulation encode to ionic and vesicular transporters, synaptic transmission and neurotransmitter, followed of changes in the expression of genes involved in cell signaling. It should be pointed out, that early expressed genes after the ischemic damage are involved in transcription and apoptosis, and they could be proposed as therapeutic target (47).

In a model *in vitro* of neurons obtained from CA1, CA3 and hippocampus region submitted to oxidative stress, a microarray analysis was made. Results showed that CA1region exhibited a high transcriptional activity in response to the oxidative stress, inflammation, metal transporters, ferroxidase and to the pre-synaptic signaling activity. While in CA3 region, the transcriptional activity was the largest to potassium and calcium channels demonstrating that each cerebral region makes a different response in the same ischemia condition and the damage shall depend of their vulnerability (48).

Mitsios in 2007 studied the gene expression with microarray technology in rat brains submitted to 1 hour of arterial occlusion with several days of sanguineous reperfusion (1 hour to 21 days) and in humans with 6 hours after death (ischemia model) and 2 to 37 days of survival (sanguineous reperfusion) and found the reduction of the gene expression of 335 genes in rat and 126 genes in human; 393 genes coincided with changes of the expression in both study systems. Whereas 184 genes changed their expression only in the rat and 36 genes changes only in human and 41 genes were down regulated in both cases, this study demonstrates a qualitative and quantitative analysis among models Used.

It has been observed that genes with changes in their expression in the ischemic process are involved in transcription, apoptosis, inflammation and neuroprotection (13, 45, 50). The most representative are shown in table 1. Mitsios (49) analyzed 13 reports, where the microarray technology was applied for the *ictus* study. These experiments were made in the ischemic model in rats, in *post mortem* cerebral human tissue and blood allowing an analogic comparison to predict the probability when the ischemic event will occur and validating the animal model to study the VCD.

115, 347 genes have been analyzed with microarray technology, expressed genes with distinct ischemic condition, term of time and with or without sanguineous reperfusion. In these studies, around 28 molecules were selected to be studied in detail, and in all cases, it was confirmed the change of gene expression and their location with *in situ* hybridization. With respect to the protein codified by those genes, they were analyzed with different technics as Western blot, immunohistochemistry, RT-PCR, immunofluorescence, northern blot and others to find their function in cells with

different experimental hypoxia, ischemic and sanguineous reperfusion conditions (11, 49).

Recently, the changes in genetic expression with microarray technology were analyzed in anterior and posterior mice, the intensification of the changes after 3 hours of hypoxia and 1 hour of oxygenation were observed. The increased genes are involved in the hypoxic response in posterior brain, and the same genes diminished in the anterior brain. Specifically, 1241 genes were regulated for the hypoxia in the cerebellum of which 642 have at least a binding site to the hepatic 4A receptor (HNF4A) and 381 have at least two binding sites to the same factor in their promoters, indicating that HNF4A is the principal transcription factor for response to hypoxia in the brain (51). In addition, it is known that this factor regulates the erythropoietin expression of which its neuroprotector effect in the ischemic damage is well known (52). The differences of gene expression in the ischemia response in anterior and posterior brain, could make a relation among the suppression of cognitive functions in anterior brain and to activate the posterior brain functions to activate the survival functions (51).

THE IMPORTANCE OF THE ISCHEMIC PRECONDITIONING IN BRAIN

The ischemic preconditioning (IPC) in brain, is a phenomenon where the neuroprotection is developed versus the ischemia, reducing the damage and neuronal death produced by ischemia. The IPC is induced exposing the cerebral tissue to other stressing factors in a sublethal way (12, 22). The protector effect of this process was described initially in the cardiac tissue and posteriorly, it has been observed in other human organs and other organisms (53, 55). It has described two kinds of protection that are variable in their temporality and in their molecular mechanisms, the early and late IPC.

The effect of early IPC emerges in the first minutes of reperfusion and it lasts for2 to 3 hours. The late IPC, is visible after 12 to 24 hours after reperfusion and its effect lasts for 2 to 3 days. The protector effects of early IPC are independent of the proteins synthesis, the effects of late IPC depend on gene activation and the production of new proteins (53, 56).

El IPC is present to respond to ischemic and sanguineous reperfusion cycles rapidly generating the release of adenosine to the extracellular space seconds after the large ischemic process started; this is an ATP product derived from its incision, it works like a signal to induce an intracellular message to activate the effector protection mechanism. The adenosine realizes its physiological effects through its interaction whit purinergic receptor A1, A2a, A2b and A3. The A1 and A2 have been implicated in the myocardium and liver IPC respectively (54). The response of second cellular messengers associated to the phospholipases C and D (PLC and PLD) and other systems associated to its activation like protein kinase C (PKC), amplify the signal activating transcription factors that activate the responses to the late IPC. One of the activated transcription factors is NFκB, of which its translocation to nucleus and activates genes that codify the super oxide dismutase that is dependent of Mg (MgSOD), the inducible nitric oxide synthase (iNO) and the heat shock proteins (HSP) (figure 3) (54, 57).

Genes that increase their expression during IPC include heat shock protein families (HSP70, HSP27, HSP90, HSP60/HSP10 and HSP32/HO-1), proteins involved in translational signals (P38 MAPK, SMAD1, guanylate cyclase, retinoic acid

receptor and the PLD) (58), transcription factors (B silencer factor, C/EBO related with TF, c-Jun y neuro-D), regulator proteins of ionic homeostasis (channels of K+, Na+ y Ca+) and proteins involved in plasticity (TGFα, TGFβ1, TGBβ receptors, caspase 3, LICE, Bcl2 and BID) (57), all involved in the activation of cell signaling pathways related directly or indirectly due to ischemic damage (26).

GENOMICS OF THE ANGIOGENESIS IN CEREBRAL ISCHEMIA

The angiogenesis is a mechanism to generate new sanguineous vessels from preexisting ones (50, 59). The cerebral ischemia induces angiogenesis during the first hour that is present. The new created vessels will be the support to the new generated cells (60) and its induction could be part of a treatment after an ictus in patients (61). Then, it is so important to study the changes in genetic expression to induce or to inhibit angiogenesis (50), to analyze its regulation and their functional products (62, 63). The malformation of new vessels, besides the vulnerability of the preexisting due to ischemic damage could cause a cerebral hemorrhage increasing the damage; it shall depend directly on the passing of time after the ischemic event and the start of treatment (59, 64).

After cerebral ischemia, the expression of multiple genes involved in the angiogenesis is activated and are shown in the 2 (64, 67). This activation has been observed in brain mice within 1 hour of ischemia where 42 genes related with angiogenesis increased their expression, of which 29 maintain their expression high for 24 hours, and 13 are activated for 21 days after ischemia (64) unchaining the formation of new sanguineous vessels (2, 50).

The therapeutic induction of angiogenesis in patients with *ictus* could be realized with related growth factors, treatments with stem cell or drugs with a specific design (39, 50, 62, 68). In any case, the angiogenesis stimulation could be the beginning of an efficient neurogenesis (62, 69), but at the same time, an intense and early stimulation should be avoided, because it could generate abnormal structures in the sanguineous vessels inducing the origin or to aggravate a cerebral edema or hemorrhages in the affected zones (59, 64).

GENOMICS OF INFLAMMATION IN THE CEREBRAL ISCHEMIA

During the cerebral ischemia an inflammatory process occurs and it could cause a rupture of the sanguineous vessels causing cerebral edema. There are genes activated specifically in the inflammatory process and their inhibition is studied to observe if it contributes to diminish the damage in the ischemic cerebral zone (6, 24, 70, 71).

The gene expression involved in inflammation has been analyzed in Wistar rats after experimental subarachnoid hemorrhage and with 24 hours of medial cerebral artery occlusion. The analysis of the mRNA in these models showed the increase in the expression of IL6, iNOS, chemokine ligand 2, TNFα and IL1β (13, 24). The gene expression of protein related with the extracelular matrix like MM9, MMP2 and MMP13 (metalloproteinases 9, 2 and 13) (72, 73). These are induced in the cerebral arteries after the cerebral ischemia through the three intracellular pathways MAPKs; p38, ERK 1/2 and SAPK/JNK. These pathways are activated in the ischemia with phosphorylation activating the transcription of factor ATF2, Alk1 and c-Jun, then, the gene expression related with

the extracellular matrix in the walls of the cerebral arteries (72), with the modification of the artery walls allow the formation of new sanguineous vessels that finally, shall permit nutrients and oxygen disposition to the ischemic tissue.

Among the activated transcription factors for the hypoxia, related with the inflammation, are the NFκB, hypoxia inducible factor 1(HIF1), interferon regulatory factor, among others. These factors activate the cytokines expression like TNFα, interleukins or the interferon, that induce the oxide nitric synthase in astrocytes and microglia cells, originating more free radicals besides the generated due to the hypoxia (13, 74). In the microglia, an explosion of proinflammatory cytokines occurs. At the same time, these cytokines act directly in endothelial cells and cause an increase in the permeability of the blood brain barrier. This process makes possible that the involved cell in the immune response, located in peripheric blood, can connect with the epithelium, to cross the blood brain barrier and finally, to migrate to the cerebral tissue affected due to the ischemia, where they shall make their function. On the other hand, it has been shown that transcription factors p53, PPAR (peroxisome proliferator-activated receptors), interferon regulatory factor 1 (IRF1), the signal transducer and activator transcription 3 (STAT-3) and the nuclear factor NFκB promote the expression of inflammatory genes that cause grave neuronal damage (70).

Finally, the increase of gene expression of IL6, TNFα, IL1β, MIP1α, MCP1, CXCL1, CXCL2, CCL20, MMP9, MMP13, VCAM1 e ICAM1, COX2, iNOS, C3 of complement HIF1, Egr1, C/EBPβ, PPARγ, IRF1, STAT3, ATF3 and their subsequent increase in the protein level they codify, suggest that inflammatory response besides angiogenesis, could explain the reorganization occurred in cerebral arteries after a cerebral attack, where there are genes that codify for proteins involved in cellular adhesion and immune response (23, 62, 70, 72, 73, 75, 76).

CONCLUSIONS

The tissue response in the presence of the ischemic event, shall depend on the intensity of the immediate damage, the time passed when the hypoxia starts, cellular type (where the neurons are the most vulnerable to the ischemia process) will depend ofnstimulus that they will receive directly or indirectly from other cerebral regions with different damage intensity or from the intact cerebral regions. All these factors shall cause the expression of only a corresponding part of the genome, according to the necessary role to execute in the affected cells in the presence of the ischemic event, translation or not of the genes to proteins. On the other hand, in the CVD neuroprotector genes are expressed to resist the ischemic damage and to induce the cellular reparation, but also genes that delete damaged cellular structures or the whole cell to avoid the sequential cellular death are expressed. At present, a variety of molecular mechanisms originated by the hypoxia or ischemia have been described; one of them is the genetic expression (39), allowing the origin of the postgenomic, where it is so important to complement the knowledge of the changes in genetic expression (63) in the presence of CVD to correlate with the functional proteomics proposing future therapeutics approaches and improve the treatments of this multifactorial disease.

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Table 1. Down regulated genes in human and rat with distinct cerebral ischemia and reperfusion conditions. The functions of several proteins than they codify are mentioned: stress response (a), receptors, transporters and channels (b) and the involved protein in the immune response (c). Modified of Mitsios, 2007 (49)

Genes	Sanguineous reperfusion time	
	Rat	Human (days)
HSP70[a]	1h- 24 h	2-6
Glucose transporter I[b]	4 h- 21 days	26-37
Aquaporin 4[b]	3 days	9-20
Signal Translator and transcriptional activator 3[b]	4 h- 3 days	9-20
Interleukin 10[c]	21 days	2-20 and 26-37
Matrix metalloproteinase 14	24 h- 3 days	2-6
Glia maturation factor beta	21 days	2-6
Proto-oncogene c-jun	1 h -24 h	9-20

Table 2. Genes up regulated in the angiogénesis process due to cerebral ischemia in mouse.

Genes	Role in the angiogenesis with cerebral ischemia
VEGF (Vascular endothelial growth factor)	The link of VEGF to its receptor on the endothelial cells surface, activates intracellular tyrosine kinases and signals to start the angiogenesis are activated.
VEGF receptor (Vascular endothelial growth factor receptor)	The angiogenesis effects of VEGFR-1 (flt-1) and the VEGFR-2 (flk-1) occurs when they are dimerized to transmit de signals of VEGF.
PDGF-βand PDGFR-β (Platelet-derived growth factor and its receptor)	It could act in the endothelial cells and pericytes interaction, allowing the vascular reorganization.
TGF-β (Transforming growth factor beta)	It regulates the cellular proliferation and differentiation. The mechanisms are not well known.
FGF-1 and FGF-2 (Fibroblast growth factor 1 and 2)	The FGF-1 and FGF-2 expression is simultaneously than angiogenesis.
Del-1 (Developmental endothelial locus-1)	It stimulates the angiogenesis through the integrins signaling pathways.
ANG 1 and 2 (Angiopoietin 1 and 2)	They regulate the vascular permeability and are associated to the angiogenesis progression through tie-2 receptor.

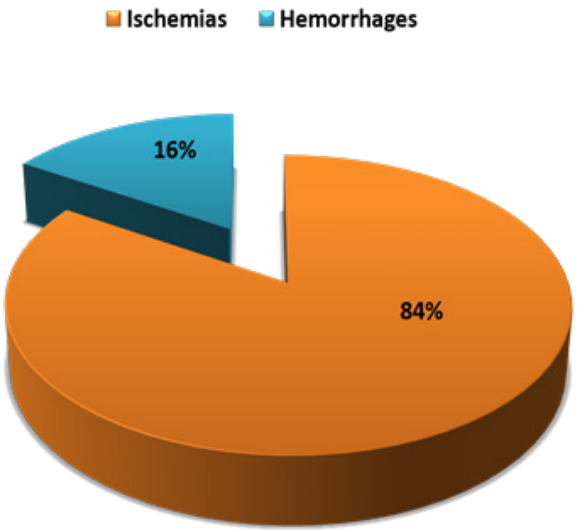

Figure 1. Frequency of the cerebrovascular disease types. Is presented as cerebral ischemic cerebrovascular disease in a proportion of the 85% and as hemorrhagic cerebral disease with a proportion of the 15%.

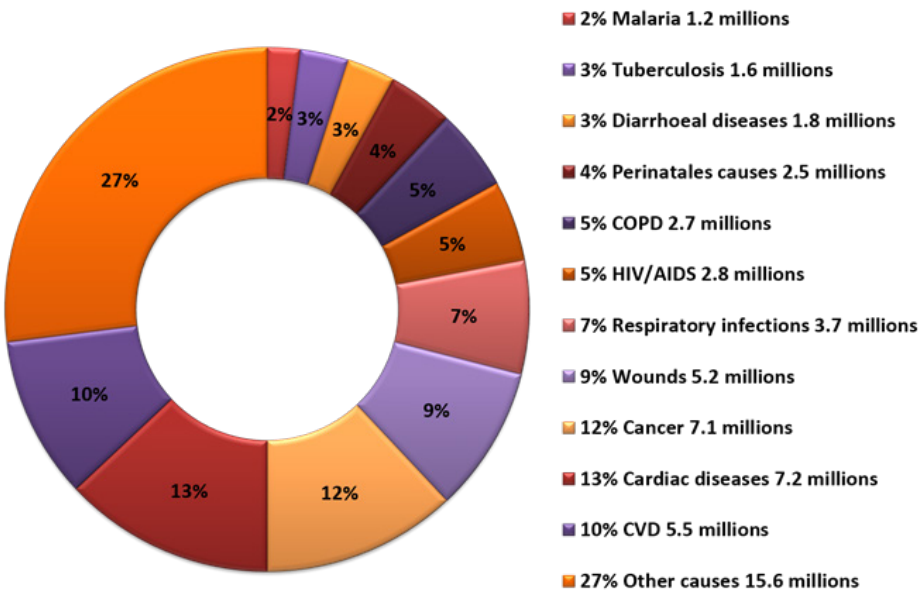

Figure 2. Frequency of death due to CVD and other main causes around the world (7).

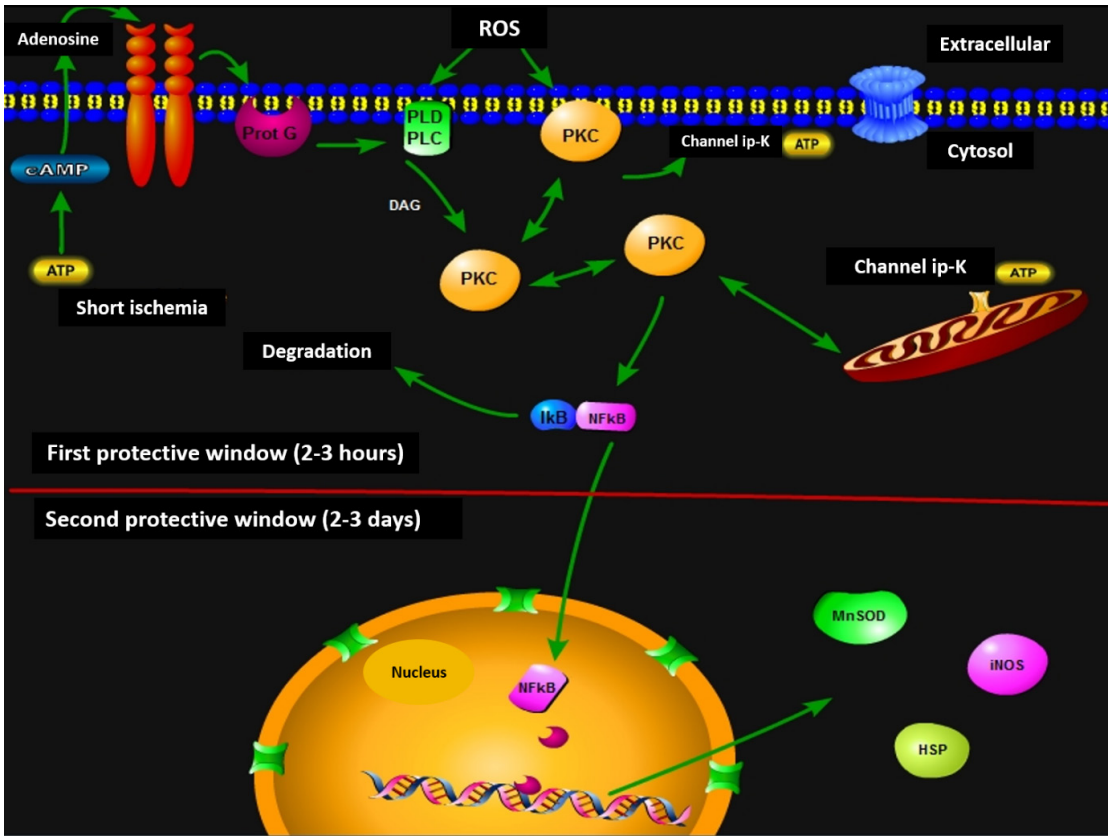

Figure 3. Molecular basis of the ischemic pre-conditioned. The corresponding mechanism to induce the first and second protection windows are showed. The adenosine level increase due to the ischemia and activate the adenosine receptors. These activate the phospholipases activity and then, the increase of the diacylglycerol (DAG) it promotes the protein kinase C (PKC) into cellular membrane, causing several reactions no yet so clear activating the transcription factors and finally, the transcription of genes that codify to antioxidant enzymes (MgSOD), heat shock proteins (HSP) and for the inducible nitric oxide synthase (iNOS). Modified of Korthuis, 1998 (53).