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Effects of Insulin-Like Growth Factor-1 (IGF-1) Receptor Signaling on Rates of Apoptosis in Retina of Dopamine Beta Hydroxylase (*Dbh*^{-/-}) Knockout Mice

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Abstract

We have investigated whether insulin-like growth factor-1 (IGF-1) receptor signaling alters rates of apoptosis in dopamine beta-hydroxylase (*Dbh*^{-/-}) knockout mice. Retinal lysates from *Dbh*^{-/-} and their heterozygote littermates (*Dbh*^{+/-}) were used to examine the role of norepinephrine in the regulation of IGF-1 receptor signaling and apoptosis in the retina. Western blot analysis was done for protein levels of total and phosphorylated IGF-1 receptor, insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), and Akt. A caspase 3 ELISA and dopamine ELISA were done on retinal lysates. To verify which regions of the retina were undergoing apoptosis, TUNEL labeling was performed. No changes in dopamine were noted between the KO and heterozygote mice. IGF-1 receptor phosphorylation was significantly decreased in *Dbh*^{-/-} mice as compared to their heterozygote littermates ($P < 0.05$ vs. heterozygous mice). IRS-1 protein phosphorylation was significantly decreased in KO mice ($P < 0.05$ vs. heterozygous mice), while no significant changes were noted in IRS-2 protein phosphorylation. Akt protein phosphorylation was also reduced in the KO mice, likely leading to increased cleaved caspase 3 levels. The increase in apoptosis in the *Dbh*^{-/-} mice occurred predominantly in the inner retina. Our results suggest that IGF-1 receptor signaling is reduced in the retina of mice with dysfunctional adrenergic receptor signaling. The data also indicate that IGF-1 receptor signaling occurs primarily through IRS-1, rather than IRS-2. The reduction in Akt phosphorylation, likely through reduced IGF-1 receptor signaling, could explain the increase in cleaved caspase 3, leading to apoptosis. These results suggest that alterations in adrenergic receptor signaling modulate IGF-1 receptor signaling, which can regulate apoptosis in the retina.

Keywords

Retina; dopamine beta-hydroxylase; apoptosis; IGF-1

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1.0 Introduction

Dopamine beta hydroxylase (*Dbh*) catalyzes the conversion of dopamine to norepinephrine, the third step of catecholamine biosynthesis (Stewart and Klinman 1988). *Dbh* is localized in all noradrenergic and adrenergic cells of central neurons of the central nervous system, sympathetic ganglia, and adrenal medulla. Sympathetic nerves typically use norepinephrine as their endogenous neurotransmitter.

Dopamine beta hydroxylase knock out (*Dbh*^{-/-}) mice offer a new approach to understanding the role of norepinephrine in development, physiology and behavior. Altered plasma *Dbh* activity has been reported in a variety of psychiatric and neurological disorders. Weinshilboum and Axelrod (1971) have found that the treatment of rats with 6-hydroxydopamine, a drug which destroys sympathetic nerve terminals, leads to a decrease in serum dopamine-beta-hydroxylase activity (Weinshilboum and Axelrod 1971). Norepinephrine is also essential for mouse fetal development, since *Dbh*^{-/-} embryos have significantly higher mortality rates (Thomas et al. 1995). Cardiovascular pressor responses are dependent on the presence of endogenous norepinephrine stores (Liles et al. 2007). Dopamine has been reported to both inhibit and enhance the release of norepinephrine and to activate alpha- and beta-adrenergic receptors and dopamine receptors. Dopamine induces norepinephrine release, producing stimulation of beta-adrenergic receptor in guinea-pig Langendorff-perfused hearts and isolated cells from right atrium (Habuchi et al. 1997). Norepinephrine, the predominant neurotransmitter of sympathetic nervous system, also has been reported to induce proliferation, hypertrophy, and migration of thoracic aorta smooth muscle cells of Sprague-Dawley rats (Xin et al. 1997; Zhang and Faber 2001; Zhang et al. 2002). Recently, we have shown that the *Dbh* KO mouse has many retinal features similar to those observed in diabetic retinopathy, including an increase of basement membrane thickening, increased numbers of pericyte ghosts, and fewer cells in the retinal ganglion cell layer (Steinle et al. 2009). Sympathectomy (surgical removal of the superior cervical ganglion) produces similar increases in basement membrane thickening and pericyte loss in the retina (Wiley et al. 2005). Therefore, it is clear norepinephrine levels can regulate a number of physiological processes within the retina.

The actions of noradrenergic receptors on insulin growth factor-1 signaling have not been explored. Insulin-like growth factor 1 (IGF-1) receptor is a member of the membrane receptor tyrosine kinase family that is involved in the transduction of the metabolic signals (Rosenfeld and Roberts, 1999). IGF-1 action is initiated through the binding to and activation of its cell-surface receptor. Activation of the IGF-1 receptor triggers intracellular events that result in cellular growth and differentiation. IGF-1 is a polypeptide growth factor with a variety of functions in both neuronal and non-neuronal cells. IGF-1 plays anti-apoptotic and other functions by activating multiple signaling pathways, including Akt kinase, a serine/threonine kinase essential for cell survival. Others reported that IGF-1 induces the activation/ phosphorylation of Akt kinase and cyclic AMP response element-binding protein (CREB) by activating different signaling pathways in PC12 neuronal cells (Zheng and Quirion 2006).

One mechanism for the cellular growth and differentiation promoted through IGF-1 receptor phosphorylation is activation of insulin receptor substrate (IRS) complexes. IRS proteins phosphorylate phosphatidylinositol-3-kinase (PI3K), which in turn, can activate Akt. Akt plays a critical role in controlling the balance between survival and apoptosis.

Since we have previous data indicating that adrenergic receptor signaling is protective to the retina through prevention of apoptosis, the goal of the present study was to determine whether this occurs through modulation of IGF-1 receptor signaling.

2.0 Material and Methods

2.1 Animals

Twelve *Dbh*^{-/-} and 12 *Dbh*^{+/-} (heterozygous) mice (3 litters) generated on a hybrid 129/SvCPJ and C57BL/6 background were used for these studies. To generate the *Dbh* KOs, *Dbh*^{-/-} males are mated with *Dbh*^{+/-} females, yielding approximately equal numbers of *Dbh*^{-/-} and *Dbh*^{+/-} pups. *Dbh*^{+/-} females were mated with *Dbh*^{-/-} males and treated with 100 ug/ml each of phenylephrine and isoproterenol (Sigma, St. Louis, MO) from embryonic day (E) 9.5 to E16.5 and 1mg/ml of L-threo-3, 4-dihydroxyphenylserine (L-DOPS, Sumitomo Pharmaceuticals, Osaka, Japan) from E16.5 to birth in the maternal drinking water to enhance fetal survival of the *Dbh*^{-/-} mice (Thomas et al. 1995). Norepinephrine or epinephrine is not essential for survival postnatally, however, so litters were not treated after birth. Sex-matched, littermate *Dbh*^{+/-} mice were used as controls because tissue content of norepinephrine or epinephrine is normal in these mice and no phenotypic differences have been observed in comparison with wild-type mice (Thomas et al. 1998). Thus, the *Dbh* KO mouse is a model of partially restored adrenergic signaling prenatally and absent adrenergic signaling postnatally. Of note, both the *Dbh*^{+/-} and *Dbh*^{-/-} fetuses are treated with agonists prenatally. Therefore it is unlikely that deficiencies found in the *Dbh*^{-/-} mice are due to adrenergic overstimulation during prenatal development. However, deficiencies in *Dbh*^{-/-} mice could be due to insufficient adrenergic signaling prenatally and/or postnatally.

Mice were killed humanely at 5 months of age by an overdose of a xylazine/ketamine mixture. All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Tennessee Health Science Center and the University of Pennsylvania and followed NIH guidelines.

2.2 Western Blot analysis

Western blot analysis was done as described previously (Steinle 2007). Primary antibodies to IGF-1 receptor (1:500, Cell Signaling, Danvers, MA), phosphorylated IGF-1 receptor on Tyr 1135/1136 (1:500, Cell Signaling), total IRS-1 (1:500, Cell Signaling), total IRS-2 (1:250, Biosource), phosphorylated Akt on serine 473 (1:500 Cell Signaling) and total Akt (1:500, Cell Signaling) were applied overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase utilized were anti-mouse (1:5000) and anti-rabbit (1:5000; Promega, Madison, WI). Antibody-antigen interactions were visualized using enhanced chemiluminescence (ECL Reagent; Amersham Biosciences, Little Chalfort, England). Densitometric analysis was carried out using Kodak Image Station 4000MM. Data is expressed as mean densitometry in arbitrary units (A.U.) and in the case of phosphorylated protein, data is expressed as a ratio of phosphorylated protein levels to total protein levels in arbitrary units. Using Prism software, mean densitometry numbers or the ratio of mean densitometry of phosphorylated protein to total protein, were used to compare data from *Dbh* KO and control (heterozygous) mice with *P*<0.05 being accepted as significant.

2.3 Immunoprecipitation

Once protein was collected into lysis buffer and assayed for protein content, 500ug of total protein lysate was combined with 40ul (1:250 dilution) of IRS-1 or IRS-2 antibodies. After washing, protein A/G beads (Santa Cruz) were added, and the immune complexes were allowed to bind at 4°C overnight. The following day, unbound proteins were removed by washing the beads, leaving the purified antibody-antigen complexes bound. 20ul of sample buffer was added to the bead sample and boiled at 100°C for 7 minutes, spun briefly, and loaded onto 4-12% tris-glycine gels for Western blotting. Blots were probed with 4G10 phosphotyrosine antibody (1:250, Millipore, Billerica, MA) to detect phosphorylated tyrosine residues within IRS-1 or IRS-2 protein complexes.

2.4 ELISA analyses

An ELISA for active caspase 3 (Cell Signaling, Danvers, MA) was done according to manufacturer's instructions to evaluate active caspase 3 levels on retinal lysates from the *Dbh*^{-/-} and heterozygous animals. Enzyme immunoassay for the quantitative determination of dopamine was done according to manufacturer's instructions (Dopamine Research ELISA, Rocky Mountain Diagnostics, Colorado Springs, CO). For this assay, dopamine is extracted by using cis-diol-specific affinity gel, acylated and then derivatized enzymatically-two independent experiment analysis. For all ELISA analyses, equal protein amounts were loaded into each well, allowing for comparisons using optical density (O.D.)

2.5 TUNEL Labeling

For TUNEL analyses of apoptosis, 5µm retinal sections were de-paraffinized, followed by antigen retrieval via Proteinase K. Following manufacturer's instructions (DeadEnd Fluorometric TUNEL System, Promega), rTdT enzyme was added to the tissue sections. In addition, propidium iodide was added to the reaction to stain cellular nuclei. Once slides were coverslipped using Fluoromount G, analyses of FITC (Tdt, dying cells) and TRITC (propidium iodide, nuclei) were visualized using NIKON ECLIPSE 80i fluorescence microscope with NIS elements software at 200× to give a view of all retinal layers and at 400× to localize the apoptotic cells.

3. Results

ELISA on Dopamine levels in retinal lysates

No changes in dopamine levels are observed in the retina of heterozygous and KO mice (Figure 1A).

Phosphorylation of IGF-1 receptor in *Dbh*^{-/-} mice

We used Western blotting to determine protein levels of phosphorylated IGF-1 receptor and total IGF-1 receptor in retinal lysates from *Dbh*^{-/-} mice and their heterozygote littermates using a phosphorylation-specific antibody for Tyr 1135/1136 on the IGF-1 receptor. IGF-1 receptor phosphorylation is significantly decreased in *Dbh*^{-/-} mice as compared to their heterozygote littermates (Figure 1B, $P < 0.05$ vs. heterozygous mice). Total levels of IGF-1 were not changed in the KO mice (data not shown).

Phosphorylation of Insulin receptor substrate-1 and Insulin receptor substrate-2 proteins in the retinal lysates of *Dbh*^{-/-} mice

Since IGF-1 receptor can signal via IRS-1 or IRS-2 proteins, we investigated the phosphorylation states of both using immunoprecipitation techniques. Tyrosine phosphorylation of IRS-1 protein is significantly decreased in KO mice as compared with heterozygous mice (Figure 1C, $P < 0.05$ vs. heterozygous mice). Conversely, IRS-2 tyrosine phosphorylation was not altered in KO mice when compared with heterozygous mice (Figure 1D), suggesting that IGF-1 receptor signaling in the retina is reduced in animals with disturbed adrenergic receptor actions primarily through reduced IRS-1 activity.

Effect of Akt protein phosphorylation on cleaved caspase 3 levels in the KO mice

In addition to observing a reduction in IGF-1 receptor and IRS-1 phosphorylation in the *Dbh*^{-/-} mice, we also observed a significant decrease in Akt activity (Figure 2A, $P < 0.05$ vs. heterozygous mice). The reduction in Akt phosphorylation, likely through reduced IGF-1 receptor signaling, could explain the increase in cleaved caspase 3 activity (Figure 2B, $P < 0.05$ vs. heterozygous mice). To verify the cellular regions of cell death, TUNEL labeling was observed more frequently in the inner retina of the KO mice versus the heterozygote littermates

(Figure 2C regions C, D vs. A, B). These results suggest that loss of adrenergic receptor signaling regulates downstream effects of IGF-1 receptor activity to increase apoptosis in the retina.

4.0 Discussion

The present study was designed to test the hypothesis that loss of norepinephrine can modulate IGF-1 receptor signaling in the mouse retina. Numerous studies have investigated whether the presence or absence of *Dbh* can influence the activity of vital organs and facilitate adaptation to physical and metabolic challenges, including cardiovascular function, bronchodilation, thermogenesis, glucose and lipid metabolism (Landsberg 1992). Very little is known about the regulatory role of *Dbh* on the IGF-1 receptor signaling pathway in the retina. The *Dbh* mouse model is a well-characterized model for loss of adrenergic signaling. Fetuses require adrenergic (primary beta1) signaling prenatally for survival (Thomas et al. 1995). Evidence suggests that this is to maintain cardiac output during periods of relative hypoxia that likely occur secondary to spontaneous uterine contractions (Chandra et al. 2006). Restoring fetal norepinephrine release using the norepinephrine precursor L-DOPS (administered via the maternal drinking water) rescues fetal survival (Thomas et al. 1995), and this can be mimicked by replacement of L-DOPS with agonists such as isoproterenol (Portbury et al. 2003; Thomas et al. 1995). Dose-survival data have been collected for both of these compounds, and close to the minimal fully effective dose is used during prenatal treatment (1mg/ml for L-DOPS and 0.1mg/ml each for isoproterenol and phenylephrine in the maternal drinking water). Levels of these drugs in the murine fetal circulation are of course unknown. However, 1mg L-DOPS in the drinking water restores fetal tissue levels of norepinephrine to ~10% of normal, and this is sufficient for rescue (Thomas et al. 1995). Thus, the *Dbh* KO mouse is a model of partially restored adrenergic signaling prenatally and absent adrenergic signaling postnatally. Of note, both the *Dbh*^{+/-} and *Dbh*^{-/-} fetuses are treated with agonists prenatally. Therefore it is unlikely that deficiencies found in the *Dbh*^{-/-} mice are due to adrenergic overstimulation during prenatal development. However, deficiencies in *Dbh*^{-/-} mice could be due to insufficient adrenergic signaling prenatally and/or postnatally. While norepinephrine and epinephrine have not been measured in the retina specifically in *Dbh* KOs, plasma and many other tissues and brain regions have been examined in adult *Dbh* KOs and, as predicted, norepinephrine and epinephrine are absent from all of them (Thomas et al. 1998).

It is well established that diabetes causes impairment of adrenergic receptor actions in a variety of organs of animals and human subjects (Schmidt 2002). Sympathectomy (surgical removal of the superior cervical ganglion) can lead to a plethora of vascular and neural changes in the rat retina (Steinle 2007; Steinle et al. 2005; Walker and Steinle 2007; Wiley et al. 2005). Recently, Steinle et al (2009) have shown that the *Dbh* KO mouse has many retina features similar to diabetic retinopathy, including apoptosis of cells in the retinal ganglion cell layer, increased retinal basement membrane thickening, and increased numbers of pericyte ghosts (Steinle et al. 2009). Taken together, these findings suggest that maintenance of adrenergic receptor signaling is beneficial to the retina. The mechanism is less clear. One possibility is that norepinephrine can modulate IGF-1 receptor activities to slow apoptosis in the retina. Our results suggest that IGF-1 receptor signaling is reduced in mice with dysfunctional adrenergic receptor signaling, due to knockout of the dopamine beta hydroxylase gene, which would result in a systemic loss of norepinephrine in the mice.

Since little has been done to investigate norepinephrine regulation of apoptosis in the retina, work on other tissues may offer some insight. Norepinephrine induces a progressive decrease in coronary flow and cardiac performance, which might result from increased apoptosis (Fu et al. 2006). Furthermore, norepinephrine induced PC12 cell apoptosis through a mitochondrial death pathway that blocks signals from the phosphatidylinositol-3-kinase (PI3K)/Akt survival

pathway (Mao et al. 2006). G-protein mediated signaling cascades can participate in the dopamine/norepinephrine-induced neuronal apoptosis in human brain (Chan et al. 2007). Therefore, much of the literature suggests that norepinephrine can induce apoptosis in a number of organs. Our present results are in sharp contrast to this literature, since our data suggest that norepinephrine prevents apoptosis in the retina. The reasons for this discrepancy might be, in part, due to the delicate and sensitive nature of retina. Furthermore, the function of sympathetic nerves in the retina may be very different than other organs, as retinal blood flow is autoregulated and under high levels of oxidative stress and light. For these reasons, norepinephrine may be protective to the retina, which is supported by our previous work indicating that sympathectomy and loss of Dbh enzyme production in the Dbh KO mice have deleterious effects on the retina (Steinle et al. 2009; Wiley et al. 2005).

While our results indicate that norepinephrine is protective to the retina, the cellular mechanism is not clear. Based on the present results, it appears that norepinephrine levels regulate IGF-1 receptor signaling, which should normally reduce apoptosis. Norepinephrine does not appear to regulate total levels of IGF-1 in the retina, since we have shown that total IGF-1 levels are not changed between the KO and the heterozygous mice (data not shown), but rather that IGF-1 receptor phosphorylation and signaling are altered. Available data strongly indicate that IGF-1 receptor has an influence on the growth of different cell types. IGF-1 receptor signaling prevents caspase activation and mitochondrial events leading to apoptosis in human neuroblastoma (Leininger et al. 2004). Others have shown that IGF-1, along with erythropoietin, is neuroprotective via activation of the PI3K-Akt pathway and inhibition of apoptosis in rat cerebrocortical neurons (Digicaylioglu et al. 2004). These results all are in general agreement with our findings. Apoptosis in our model occurs predominantly in the inner retina. In addition to that associated with the ganglion cells, some labeling may also be associated with the cytoplasm of the Müller cells; however, additional labeling is required to verify this proposition. Nonetheless, our data match work in other systems to indicate that normal IGF-1 receptor signaling is anti-apoptotic, and its decrease can produce increased apoptosis.

Phosphorylation of IGF-1 receptor leads to increased phosphorylation of insulin receptor complexes (IRS), which in turn can phosphorylate Akt (Rosenfeld and Roberts, 1999), a downstream molecule of insulin receptor substrate. The phosphorylation of Akt activates anti-apoptotic factors, such as Bcl2, leading to a general decrease in the cleavage of caspase 3 (Rosenfeld and Roberts, 1999). Dromard M et al. (2007) have shown that decreased phosphorylation of insulin receptor substrate-1 by PTPL1, a tumor suppressor gene in breast cancer cells, induces apoptosis through IRS-1/PI3K/Akt signaling pathway, thus inhibiting the IGF-1 effect on cell survival (Dromard et al. 2007). A polysaccharide that inhibits cell proliferation and induces apoptosis in AGS gastric cancer cells works through inhibition of IGF-1 receptor signaling and the PI3K/Akt pathway (Kwon and Nam 2007). While it is clear that IGF-1 receptor signaling can regulate apoptosis through Akt phosphorylation, little research has been done to correlate norepinephrine with changes in the IRS complexes directly. The present data suggests that this may be a new area for investigation.

5.0 Conclusions

In conclusion, the data indicate that norepinephrine regulates IGF-1 receptor phosphorylation. IGF-1 receptor signaling in the retina occurs primarily through phosphorylation of insulin receptor substrate-1, rather than insulin receptor substrate-2. The decrease in phosphorylation of insulin receptor substrate-1 leads to a reduction in activity of the PI3K/Akt cascade. The reduction in Akt phosphorylation, likely through reduced IGF-1 receptor signaling, could explain the increase in cleaved caspase 3 and TUNEL labeling in the inner retina, since IGF-1 is a known survival factor. These results are the first to our knowledge to link adrenergic

receptor actions and IGF-1 receptor signaling in the retina. These results suggest that loss of adrenergic receptor signaling causes decreased IGF-1 receptor signaling, producing increased apoptosis in the retina. These findings suggest that restoration of adrenergic receptor signaling may be a new target for therapeutic intervention to inhibit the early phases of diabetic retinopathy.

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Figure 1.

Figure 1A. Dopamine levels in retinal lysates

Bar graph of Dopamine levels (ELISA) in retinal lysates from knockout mice for dopamine beta hydroxylase *Dbh*^{-/-} and their heterozygote littermates *Dbh*^{+/-}, N=7.

Figure 1B. Insulin-like growth factor-1 receptor phosphorylation in *Dbh*^{-/-} and heterozygous mice

Representative Western blots and a bar graph of the ratio of the mean densitometry values of phosphorylated insulin-like growth factor-1 receptor to total insulin-like growth factor-1 receptor. Retinal lysates were from knockout mice for dopamine beta hydroxylase *Dbh*^{-/-} and their heterozygote littermates (*Dbh*^{+/-}). P<0.05 vs. heterozygous mice, N=6.

Figure 1C. Insulin receptor substrate-1 protein levels in *Dbh* KO and heterozygous mice

Representative Western blots and a bar graph of the ratio of the mean densitometry values of phosphorylated insulin receptor substrate-1 to total insulin receptor substrate-1 protein. Retinal lysates used were from *Dbh*^{-/-} and their heterozygote littermates. P<0.05 vs. heterozygous mice, N=5.

Figure 1D. Phosphorylation of Insulin receptor substrate-2 in *Dbh*^{-/-} and heterozygous mouse retina

Western blot image and bar graph of the ratio of the mean densitometry values of phosphorylated insulin receptor substrate-2 to total insulin receptor substrate-2 protein. Retinal lysates used were from *Dbh*^{-/-} and their heterozygote littermates, N=5.

Figure 2.

Figure 2A. Changes in Akt phosphorylation in Dopamine beta hydroxylase KO mice retina. Representative blots and a bar graph of the ratio of the mean densitometry values of phosphorylated Akt to total Akt protein. Retinal lysates were from *Dbh*^{-/-} and their heterozygote littermates. $P < 0.05$ vs. heterozygous mice, $N = 4$.

Figure 2B. Cleavage of caspase 3 in Dopamine beta hydroxylase KO mouse retina. Bar graph of active caspase-3 protein (ELISA) levels from retinal lysates used from *Dbh*^{-/-} and their heterozygote littermates *Dbh*^{+/+}, $N = 4$.

Figure 2C. Apoptosis occurs in the inner retina of *Dbh*^{-/-} mice. TUNEL labeling of 5 μ m retinal sections from *Dbh*^{-/-} and *Dbh*^{+/+}. There is enhanced FITC labeling in the inner retinal regions in the KO retina (C,D) as compared to their heterozygote littermates (A,B). Some labeling may be present in Müller cell cytoplasm following ingestion of apoptotic cells. Panel A, B represents the heterozygous retina, while panel C, D represent the KO retina. Magnification was 200 $\times$, for panels A, C and scale bar is 5 μ m. Panels B and D were captured at 400 $\times$ with the scale bar equal to 50 μ m. Arrows are present to indicate cells that are TUNEL positive.