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Original Article

Evaluation of signaling molecules in the post-autotomy initiation phase of tail regeneration in the lizard *Hemidactylus flaviviridis*

Pradnya A. Deshmukh, Isha Y. Bhatt, Riddhi R. Jape, Palak N. Jivani, Hemangi R. Kavale, Vandana V. Nambiar, Sunny S. Prakashkar and A.V. Ramachandran

Division of Developmental and Reproductive Endocrinology, Department of Zoology, Faculty of Science, MS University of Baroda, Vadodara - 390 002, India

Summary

Epimorphic regeneration, as exemplified by lizard tail, involves the formation of regeneration blastema as a pre-requisite for replacement growth. The formation of blastema is preceded by many regressive changes like tissue demolition, histolysis, inflammation, wound closure, de-differentiation, cell migration and proliferation. Induction of signaling molecules would be crucial in the molecular biology of immediate post-autotomy period in providing the trigger for the initiation of regeneration. It is in this behest that the levels of the cAMP, cGMP and NO have been assayed during the first 72 hr post caudal-autotomy in the lizard *Hemidactylus flaviviridis*. The influence of exaggerated exogenous induction of NO as well as prolonged action of cGMP by the use of PDE5 inhibitor (sidenafil citrate) have also been evaluated in terms of the number of days taken to complete wound healing and form a blastema as well as the length of tail regenerated at the end of 20 days. cAMP-mediated PKA activation and downstream signaling cascades appear to be the principal mechanism as against cGMP-mediated mechanisms. Controlled NO generation appears to be of pivotal significance in minimizing apoptotic cell loss as substantiated by the reduced caspase 3 activity. Exaggerated induction of NO and prolongation of cGMP action appear to promote apoptotic cell loss resulting in retarded regenerative growth. It can be concluded from the present observations that cAMP- and PKA-mediated events occur first and that minimizing cell apoptosis by controlled NO generation is important in the formation of robust regeneration blastema.

Key words: Apoptosis, caspase 3, lizard, regeneration, signaling molecules

Introduction

Regeneration is the process by which some organisms replace or restore lost or amputated body parts. Organisms differ markedly in their ability to regenerate parts. Some grow a new structure on the stump of the old one. By such regeneration organisms may dramatically replace substantial portions of themselves when they have been cut into two, or may grow organs or appendages that have been lost. Lizards regenerate their tails as an adaptive mechanism by breaking off the original tail as a method of escape when grasped or even chased by an enemy. The process of losing a body part spontaneously is called autotomy and in the case of lizard tail it occurs at pre-existing breakage planes on the vertebral column.

The cut end of the autotomized tail seals off by the formation of a wound epithelium and the process of regeneration initiates from a mass of mesenchymal cells that accumulate below the wound epithelium. This epithelium divides and becomes multilayered and together with the accumulated mesenchymal cells form a dome-shaped projection referred to as regeneration blastema**.** The multilayered apical epithelium is referred as blastemic epithelium while the underlying undifferentiated cells are termed blastemal cells (Ramachandran, 1996). The blastemal cells are formed by dedifferentiation of mesodermal parts of the original tail comprising of connective tissues like muscle septae, adipose tissue, dermis and, perhaps, osteocytes of vertebrae. Even nomadic cells in the form of migrating lymphocytes and/or pleuripotent stem cells from systemic sources (Shah et al., 1980; Ramachandran et al., 1985) are likely to serve as the sources of mesenchymal blastemal cells. The accumulated dedifferentiated blastemal cells then proliferate and redifferentiate into cartilage, muscle, skin and other tissues in a proximo-distal direction. Inside the newly formed

Correspondence to be addressed to: Dr. A.V. Ramachandran, Ph.D. Email: avrcn@rediffmail.com

cartilagenous tube, the ependymal cells proliferate and gradually extend out providing the initial trigger for initiation of regeneration.

Ability to induce regeneration of lost or damaged tissues would greatly benefit patients suffering from loss of bodily function by organ failure, forced or accidental amputation of limbs or third degree burns. Regenerative medicine shall benefit greatly if we understand the inherent fundamental mechanisms and signaling pathways. Though initiation of regeneration is an innate process, various exogenous and endogenous factors play vital roles. During initiation of regeneration, various processes are likely to take place such as inflammation, wound repair, dedifferentiation, cell proliferation, etc. All these processes require signal transduction cascades for their effective expression. So far no studies have been undertaken on the role of signaling molecules during the initial periods of regeneration. The factors ensuring appropriate intercellular communication during wound repair are not completely understood. Although protein-type mediators are well-established players in this process, signaling molecules such as nitric oxide, cyclic AMP and cyclic GMP are some short-acting signal transduction molecules whose role(s) need to be evaluated.

Nitric oxide (NO), a pleotropic biomolecule, has beneficial effects on wound repair which can be attributed to its functional influence on angiogenesis, inflammation, cell proliferation, matrix deposition and remodeling (Cristiano et al., 2000). NO has been implicated in a variety of signal transduction mechanisms such as neural transmission, vasodialation and immunoregulation (Knowles and Moncada, 1994) under the influence of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interferon-γ (INF-γ) (Lou and Chen, 2005). Interestingly, complex interplay of these pro-inflammatory cytokines and growth factors employssecond messengers such as cAMP and cGMP for completion of the signal transduction cascades (Mladinic, 2007; Qiu et al., 2002). In particular, cyclic AMP as an important second messenger in signaling pathways regulates cellular processes involved in development and regeneration, as observed in axonal regeneration in central nervous system (CNS) (Hannila et al., 2007; Cai et al., 2001; Pearse et al., 2004; Cui et al., 2004).

The precise role of these short-acting molecules is under spatial and temporal regulation. Hence, in the present study, the level of nitric oxide, cAMP and cGMP has been estimated at different time intervals to assess their role in initiation of regeneration. In order to elucidate the exact mechanism and possible role of NO, sodium nitroprusside has been used for generation of NO together with a phosphodiesterase 5 inhibitor (sidenafil citrate) for prolonged effect of NO-mediated cyclic GMP. It is known that NO can prevent TNF-mediated activation of pro-apoptotic caspase-3 (Rai et al., 1998). Therefore, NO-mediated prevention of degree of cellular apoptosis is also assessed by expression of caspase-3 at different time intervals.

In the above context, the present study has been carried out during the initial periods of regeneration, with the following objectives:

a) Possible role of nitric oxide (NO) signaling

b) Involvement of cAMP and CGMP signaling

c) Effect of over-expression of NO

d) Effect of prolonged cGMP action

e) Degree of apoptosis

Materials and Methods

Adult lizards *Hemidactylus flaviviridis* of both sexes, weighing 10±2 g and measuring 70-80 mm snout–vent length, were procured from a local animal supplier. The animals were maintained on diet of cockroach nymphs and water *ad libitum*. Animal were kept in well ventilated wooden cages measuring 18"×15"×10" with one side made of transparent glass. Each cage housed not more than 8 lizards and was maintained at a cage temperature of 32-34° C.

Analytical grade sodium nitroprusside was procured from SRL Laboratories, Mumbai, India, and sidenafil citrate gel was obtained from Alembic Pharma, Mumbai, India, as a gift for research purpose.

Experimental set-ups

Set up-1. Measurement of cAMP:

Sixteen lizards were taken and their tails were autotomized at 8:00 hrs. The autotomized end of the tail stump of four lizards each was taken at zero, 24, 48 and 72 hr post-autotomy for the estimation of cAMP content by using Kit purchased from R & D system, USA.

Set up-2. Measurement of cGMP:

Sixteen lizards were taken and their tails were autotomized at 8:00 hrs. The autotomized end of the tail stump of 4 lizards each was taken at zero, 24, 48 and 72 hr post-autotomy for the estimation of cGMP content by using kit purchased from R & D System, USA.

Set up-3. Measurement of nitric oxide (NO):

Sixteen lizards were taken and their tails were autotomized at 8:00 hrs. The autotomized end of the tail stump of 4 lizards each was taken at zero, 24, 48 and 72 hr post- autotomy for the estimation of NO content by using Kit purchased from R & D system, USA..

Set up-4. Generation of nitric oxide:

NO free radicals were generated at local site by injecting sodium nitroprusside (1µg/g) (prepared by dissolving 10 µg of the chemical in 0.05ml of 0.6% saline) *in loco* (injected at the cut end of the tail) every 12 hr post autotomy. Treatment was continued up till blastema stage was attained. Morphometric analysis was done up till 15 days after initiation of growth. Six lizards were used to generate NO and another six lizards were used as control and received 0.05 ml of 0.6% saline at the cut end.

Set up-5. Prolongation of cGMP activity:

Sustained cGMP action was maintained by application of sidenafil citrate gel (0.5mg/animal), a PDE5 inhibitor, administered as topical application at the site of autotomy. Application was carried out every 12 hr post-autotomy till the time of blastema formation. Length of tail regenerated was measured in mm, using a meter ruler, from the time of initiation of growth till 20 days. Four lizards served as experimental and four lizards served as control.

Set up-6. Assessment of apoptosis:

Possible occurrence and degree of apoptosis was evaluated by assaying caspase3 activity in unautotomised and autotomised tails at 24, 36, 48 and 72 hr using kit purchased from R & D System, USA. Three lizards were used for assaying caspase 3 activity in the pre-autotomy state and twelve lizards, 3 for each time period, were used for evaluation post-autotomy.

Parameters assessed

Tail autotomy was performed by pinching off the tail at the third segment from the vent, in the morning. The length of tail removed varied between 55 and 65 mm. The various arbitrary stages such as wound healing, blastema formation and initiation of growth of regenerate were observed and their timing of occurrence was recorded. After initiation of growth, the length of regenerate in mm was measured using a graduated meter ruler. Data were analyzed adopting Student's *t* test.

Results

Change in cAMP and cGMP levels

The change in cAMP and cGMP levels at different hours post-autotomy are depicted in figure1. Significant up-regulation of cAMP and cGMP were recorded at 24 hr post-autotomy. Subsequently, cAMP levels remained above normal till 72 hr. cGMP level decreased through 36-72hs.

Change in nitric oxide levels

The change in nitric oxide levels are depicted in figure 2. Nitric oxide level was significantly increased from 24 to 72hr post-autotomy except for a slight decrease at 36 hr. Overall, up regulation of nitric oxide was recorded in the initial post-autotomy periods.

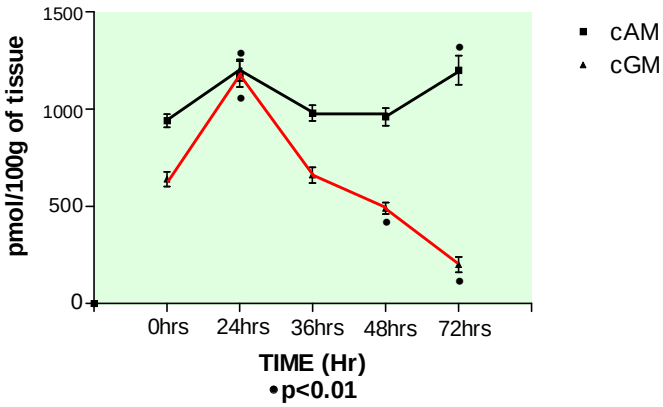

Fig. 1. Levels of cyclic AMP and cyclic GMP (pmol/100g of tissue) at different hours post-autotomy.

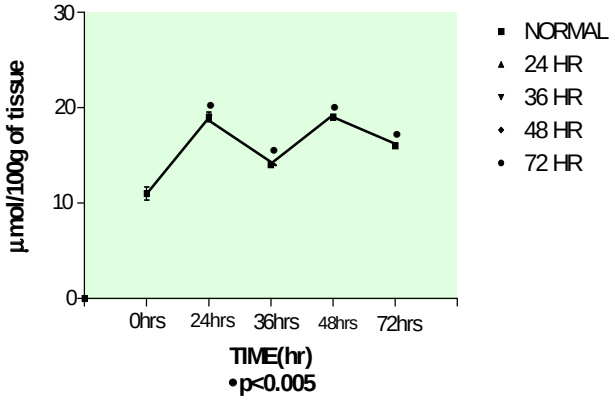

Fig. 2. Levels of nitric oxide (µmol/100g of tissue) at different hours post-autotomy.

In loco *NO generation*

The *in loco* NO generation by exogenous treatment with sodium nitroprusside did not record any significant difference with respect to the number of days

taken to attain the arbitrary stages of regeneration, like wound healing, blastema and initiation of growth (Table 1). However, length of tail replaced at the end of 20 days showed a significant decrease from 23.3 mm in control to 14.1 mm in experimental lizards (Fig. 3).

Effect of prolonged cGMP action by Sidenafil citrate

Effect of prolonged cGMP action on the attainment of arbitrary stages of regeneration is depicted in the table 2. Prolongation of cGMP action by sidenafil citrate (a PDE5 inhibitor) did not produce any effect on the number of days taken to attain the arbitrary stages of regeneration, like wound healing, blastema formation and initiation of growth. However, length of tail regenerated at the end of 20 days was significantly decreased to 19.1 mm as compared to 26.1 mm in control lizards (Fig. 4).

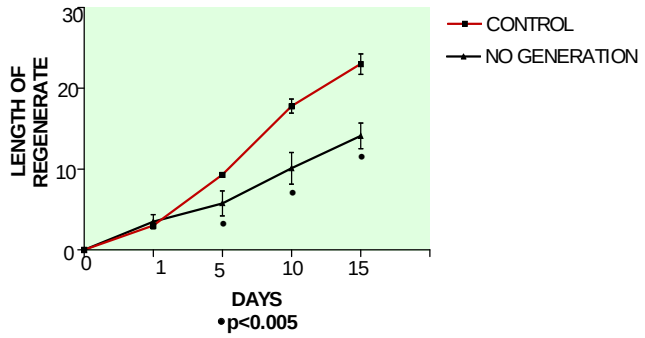

Fig. 3. Graph showing length of tail (mm) regenerated at different time periods (days) post-autotomy in control and experimental lizards.

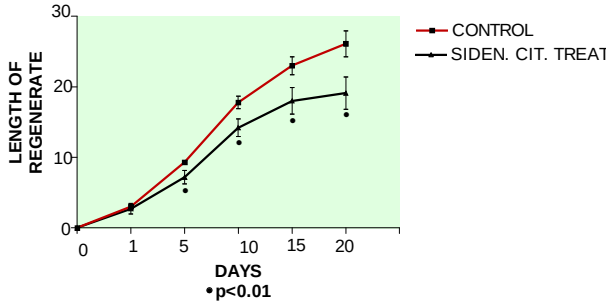

Fig. 4. Graph showing length of tail (mm) regenerated at different time periods (days) post-autotomy in control and experimental lizards.

Assessment of apoptosis

The caspase-3 activity, as shown in the figure 5, was significantly decreased by 24 hr, from which level increased to reach the normal by 72 hr.

Discussion

Elevated levels of cAMP, cGMP and NO and concomitantly decreased activity of caspase-3 were recorded within 24 hr of caudal autotomy. Both cAMP, cGMP showed temporal variation though there were subtle differences in the pattern of expression. cGMP peaked at 24 hr post - autotomy following which there was decrease to well below the pre–autotomy level till 72 hr, while cAMP tended to remain higher. The high cAMP level is well supported by similar observations in other models of regeneration such as regenerating spinal cord of opossum (Moon and Bunge, 2005; Mladinic, 2007), regenerating forelimb of newts (Taban et al., 1978; McLaughlin et al.,1983) and axonal regeneration (Qiu et al., 2002; Hannila and Filbin, 2008). One plausible explanation for the elevated cAMP level for a prolonged duration could be that cAMP, being a very important signal transduction molecule, it may be involved in triggering a multitude of signal transduction pathways related to different aspects of regeneration such as matrix remodeling, dedifferentiation and cell proliferation. Upregulated cAMP level persisting till 72 hr could be conducive for ependymal and neuronal outgrowth and secretion of neurotrophic factors by PKA-mediated cascades. Indirect analysis of cAMP has been done earlier in this laboratory by assaying the levels of activity of phosphodiesterase (PDE), the hydrolytic enzyme of cAMP. By keeping in view the reciprocal relationship with PDE, two phases of elevated cAMP content have been inferred, once during post-autotomy periods and later during the post-blastemic differentiation phase (Ramachandran et al., 1983). The present study is in consonance with the above observed trend in PDE activity and further emphasizes the therein speculated roles for cAMP in various preparative and pre-requsite events such as phosphorylation of histones, induction of ornithine decarboxylase, synthesis of polyamines, and generating precursors for and regulating DNA synthesis. However, its role in ependymal and neuronal outgrowth and induction of trophic factors may be of greater relevance in the present context considering the time period of 0 -72 hr post-autotomy, which precedes the occurrence of regeneration-specific events like tissue dedifferentiation, mobilization of nomadic cells, matrix remodeling and epithelial cell migration and proliferation, all crucial for regenerative non–scarring wound healing and blastema formation.

The first 24 hr post-autotmy represents a critical phase of inflammatory response leading to release of several proinflamatory molecules, cytokines and growth factors destined to initiate regeneration. This might account for the

elevated levels of both cAMP and cGMP at 24 hr. cGMP is a second messenger in the nitric oxide signaling pathway, and concomitant increase in cGMP, together with elevated levels of NO, implicates NO in the initial phase of regeneration i.e., the first 24 hr. The importance of NO during the initial phase of regeneration is inferred from the fact that mammalian liver regeneration has been found to be impaired in iNOS-deficient mice (Rai et al., 1998). In addition, NO production and subsequent increase in cGMP have been reported from *in vitro* studies in fibroblasts (Haby et al., 1994) and PC12 cells (Uberall et al., 1994). Inflammation is an early response to tissue injury spread over 24 hr from the onset of injury. This phase involves coordination between the immune system and the injured tissue. It is well known that small blood vessels, neutrophils, activated macrophages and T-lymphocytes infiltrate the injured tissue microenvironment (Tidball, 1995; MacIntyre et al., 2000). Wound macrophages appear to be a source of nitric oxide production in the early phase of 24 hr (Lee et al., 2001). Hence, the activation of the NO signaling pathway, as observed in the increased NO and cGMP, can be attributed at least in part to the proinflamatory microenvironment at the site of autotomy.

Table 1. Number of days taken to attain various arbitrary stages of regeneration in control and experimental lizards receiving sodium nitroprusside (1µg/g) every 12 hour post-autotomy.

Stages of regeneration	Set 1 (control)	Set 2 (sodium nitroprusside)
Wound healing	7.0± 0.0	7.0±0.40
Blastema formation	9.0± 0.0	8.5±0.28
Initiation of growth	10.0± 0.0	11.0±0.40

Another interesting observation in the present study is the trend of activity of caspase-3. Decreased caspase-3 activity at 24 hr coincides with the elevated levels of cGMP and NO. In the light of this observation, it is reasonable to assume that NO is anti-apoptotic in the initial phase of regeneration. The latter is beneficial for initiation of future events such as angiogenesis. Vascular endothelial growth factor (VEGF) is the most potent and specific growth factor for both angiogenesis and vasculogenesis and is induced by NO (Krazier et al., 2001; Sato et al., 2001; Yancopoulos et al., 2000; Ronco et al., 2007). This anti-poptotic activity of NO, coupled with the many other studies (reviewed by Kolb, 2000). Under *in vivo* condition, low level of NO forms caspase-3-*S* nitrosation product leading to inactivation of caspase-dependent apoptotic pathway (Rossig et al., 1999). The contrasting effects of nitric oxide on apoptosis are related to its concentration, flux and cell type and most importantly the redox state of cells which ultimately determine the action of NO on cell multiplication and survival (Kolb, 2000). The temporal changes observed in the present study prompts the assumption that it could be either the titer of NO or the microenvironment of the site of autotomy that is responsible for changes in the degree of apoptosis across the experimental period. Thus, the up-regulation of NO together with decrease in caspase 3 activity during the first 72 hr suggests decreased cell apoptosis and increased cell viability in the pre-blastemic period.

With the purported involvement of NO and NO-mediated cGMP in the initial phase of regeneration, a second set of experiment was performed using direct NO donor, sodium nitroprusside (SNP), in order to generate maximal level of NO at 24 hr post autotomy, the period during which the endogenous peak in NO occurred. Contrary to the anticipated hastening of the occurrence of different stages associated with regeneration and increase in the length of the regenerate, it was seen that there was no changes as far as the time taken to reach wound healing and blastema stages were concerned though there was significant delay in the post-blastemic growth of the regenerate. It is reasonable to expect an increase in the activity of caspase on the basis of the current observation despite the fact that assay of caspase activity was not carried out under the influence of NO donor.

Table 2. Number of days taken to attain the various arbitrary stages of regeneration in control and experimental lizards receiving sidenafil citrate gel (0.5mg/animal) at 12 hr intervals.

Stages of regeneration	Set 1 (Control)	Set 2 (Sodium nitroprusside)
Wound healing	7.0± 0.0	7.0±0.40
Blastema formation	9.0± 0.0	8.5±0.28
Initiation of growth	10.0± 0.0	11.0±0.40

Further degree of assertiveness in this regard arises from the result of experiments wherein the prolonged action of cGMP was induced by administration of sidenafil citrate, a known PDE5 inhibitor, which can account for the observed retardation in regenerative growth. These results point to a possible surmise that prolonged / protracted NO may be counterproductive and may become pro–apoptotic rather than anti-apoptotic. Pro-apoptotic role of NO in this connection has been reported in leukemic cells (Kuo et al., 1996; Kwak et al., 1998). Data from several earlier experiments have highlighted the dual role of nitric oxide in apoptosis and its possible mechanisms (Kolb, 2000; Zeini et al., 2005). On the basis of the results from the present

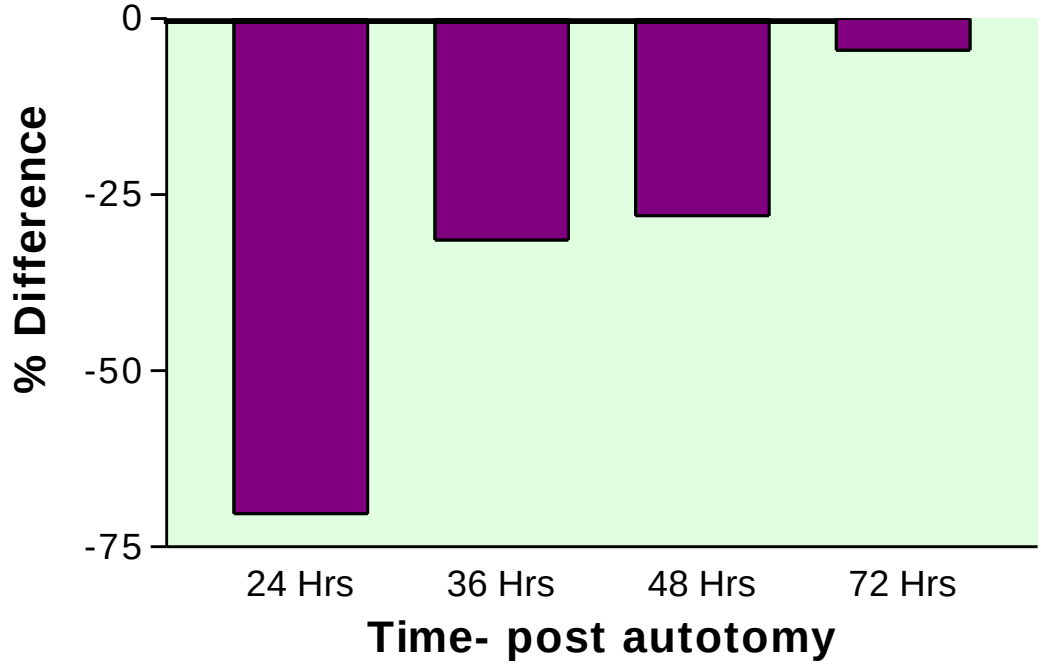

Fig. 5. Graph showing percentage change in caspase-3 activity at different hours post-autotomy.

study and on the basis of the background information from earlier work, it can be speculated that the pro- or anti-apoptotic role of NO follows two independent mechanisms. The anti-apoptotic role is through direct action of cGMP as second messenger while the pro-apoptotic role is independent of cGMP since prolongation of cGMP action by PDE5 inhibitor was unable to block the activation of apoptotic pathway. Moreover, the endogenous cGMP level was significantly decreased between 24 and 72 hr post-autotomy. It is inferable that supra-physiological level of NO generated by SNP might have led to the formation of peroxynitrile free radical there by altering the redox state of the cells leading to reactive nitrogen species (RNS)-mediated activation of apoptotic pathway.

In conclusion, it is presumed that the initial phases of regeneration are marked by decreased cell loss by apoptosis, and NO signaling pathway is involved in the initial trigger. This initial trigger is through the action of cGMP and PKG downstream of NO. However, in the absence of any known substrates for these PKG's, there is a possibility of an alternate signaling pathway independent of cGMP (Kolb, 2000). Only trace quantity of endogenous NO switches on this signaling cascade affecting the activity of caspase-3 and thereby preventing injury- / inflammation-mediated apoptosis. The level of NO in the regenerating system is apparently homeostatically controlled by a multitude of signaling molecules. However, cAMP-and PKA - mediated events seem to be of primary importance in the molecular mechanisms preceding the establishment of a regeneration blastema. An elaborate understanding of these signaling molecules, transcription factors and changes in protein profile will throw light on the precise mechanism involved and would pave way for engineering the local environment in favor of regeneration.

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