Protein abundance of vascular endothelial growth factor (VEGF) and Akt1 and VEGF gene sequences in three tissues of the African lungfish, *Protopterus annectens* aestivating in normoxia and hypoxia

Low, S. Y.¹ and Ip, Y. K.².

Department of Biological Sciences, National University of Singapore
Blk S15, Science Dr 2, Singapore 117543.

ABSTRACT

In this study, we compare and contrast the effects of aestivation in normoxia and aestivation in hypoxia on protein abundances of vascular epithelial growth factor (VEGF) and Akt1 in *Protopterus annectens* with tissues sampled on day 3 and day 6 (induction phase), or day 12 (maintenance phase). Overall, results obtained indicate that effects of aestivation in normoxia on VEGF and Akt1 in heart, kidney and intestine of *P. annectens* differed from those of aestivating in hypoxia. Specifically, aestivation in hypoxia, but not normoxia, had an effect on the VEGF protein abundance in the kidney during the induction phase on day 3. On the other hand, aestivating in normoxia, but not in hypoxia, had an effect on the VEGF protein abundance in the intestine during the maintenance phase on day 12. Since information in the literatures on aestivation in African lungfishes often do not clarify the availability of oxygen, results obtained from this study suggest that those information must be evaluated with caution. More importantly, these results indicate for the first time that aestivation could involve changes in angiogenesis in various tissues of *P. annectens*.

INTRODUCTION

Many reports in the literature describe changes in behavioral, structural, physiological and biochemical levels in aestivating lungfishes. However, evaluation of the observed phenomena is difficult as the specific phase of aestivation was often not specified. In addition, many previous studies reported in the literature did not quantify the degree of hypoxia that the aestivating animal was exposed to. The objective of this study was to compare and contrast the effects of aestivation in normoxia and aestivation in hypoxia on protein abundances of vascular epithelial growth factor (VEGF) and Akt1 in *P. annectens* with tissues sampled on day 3 (induction), day 6 (entering into aestivation) or day 12 (undergoing aestivation). This study focused on the heart, kidney and intestines of *P. annectens*. According to Icardo et al. (2008), aestivation involves a combination of upregulation and downregulation of cell activities. Therefore, it was hypothesized that the tissues of *P. annectens* would exhibit differential changes in VEGF and Akt1 protein abundances when aestivating in normoxia or hypoxia.

MATERIALS AND METHODS

Western blot was carried out on the heart, kidney and intestines of *P. annectens*. Results were presented as means ± standard error of mean (S.E.M.). Data were analyzed using one-way analysis of variance (ANOVA) with polynomial contrast and Tukey post hoc test (SPSS version 13.0) to evaluate differences between means where applicable. Differences with *P* < 0.05 were regarded as statistically significant. Partial nucleotide sequences of VEGF in heart, kidney and intestine were obtained through total RNA isolation, first strand cDNA synthesis, RT-PCR or RACE-PCR and purified extension products were subsequently analysed via automatic sequencing using 3130xl Genetic Analyzer (Applied Biosystems). Partial sequences of VEGF in the three tissues were aligned using ClustalW multiple alignment of the Bioedit Sequence Alignment Editor Software version 7.0.5.3 with default settings and a phylogenetic tree was constructed using MEGA 4.0.

¹ Student
² Supervisor
RESULTS

The protein abundance of VEGF in the heart of fish aestivating in normoxia or hypoxia for 3, 6 or 12 days were comparable with the corresponding control values of fish kept in freshwater. There was no significant change in protein abundance of VEGF in heart of control fish, fish aestivating in normoxia or fish aestivating in hypoxia throughout the 12-day period. Similarly, there was no significant change in protein abundance of VEGF in the kidney of control fish throughout the 12-day period. By contrast, there was a significant increase in the protein abundance of VEGF in fish aestivating in normoxia on day 12. The protein abundance of VEGF in the kidney of the fish aestivating in hypoxia increased on day 3 and 12. For fish aestivating in hypoxia, protein abundance of VEGF was significantly higher than corresponding value of the control fish kept in freshwater and fish aestivating in normoxia on day 3. On day 12, the protein abundance of VEGF in fish aestivating in normoxia and hypoxia were significantly higher than the corresponding value of the control fish kept in freshwater. There were no significant changes in protein abundance of VEGF in the intestine of control fish, fish aestivating in normoxia or fish aestivating in hypoxia throughout the 12-day period. However, on day 12, the VEGF protein abundance in the intestine of fish aestivating in normoxia was significantly lower than that of control fish kept in freshwater.

The protein abundance of Akt1 in the heart of fish aestivating in normoxia was comparable to that of fish kept in freshwater, and they remained relatively constant throughout the 12-day period. By contrast, there was a significant increase in protein abundance of Akt1 in the heart of fish aestivating in hypoxia on day 12. On day 3, protein abundance of Akt1 in fish aestivating in hypoxia was significantly lower than that of fish kept in freshwater. On day 6, the protein abundance of Akt1 in fish aestivating in hypoxia was significantly lower than those of fish kept in freshwater and the fish aestivating in normoxia. There were a significant decreases in protein abundance of Akt1 in the kidney of fish kept in freshwater or aestivating in normoxia on day 6 as compared to the corresponding values of fish kept in fish kept in freshwater or aestivating in normoxia on day 3. During aestivation in hypoxia, protein abundance of Akt1 increased on day 6. Consequently, the protein abundance of Akt1 in the kidney of the fish aestivating in hypoxia was significantly higher than that of the control fish in freshwater or fish aestivating in normoxia on day 6. There was no change in protein abundance of Akt1 in the intestine of fish kept in freshwater or aestivating in hypoxia throughout the 12-day period. However, aestivating in normoxia led to a significant decrease in the intestine Akt1 protein abundance on day 12. Incidentally, the protein abundance of Akt1 in the intestine of fish aestivating in normoxia was significantly lower than that of the control fish kept in freshwater on day 12.

Partial nucleotide sequences of the coding region of VEGF in heart, kidney and intestine were aligned using ClustalW multiple alignment of the Bioedit Sequence Alignment Editor software version 7.0.5.3 showed that the sequences share significant similarities in the coding region of the VEGF in the three tissues. There was a high bootstrap support (100%) for the positioning of the VEGF of the heart of P. annectens with that of the clade that consists of the kidney and intestine of P. annectens. This was supported by a high sequence identity between the sequences. VEGF from P. annectens appeared to cluster more closely with Xenopus laevis instead of the fishes and the higher vertebrates.

DISCUSSION

During aestivation in normoxia, P. annectens was exposed to air with 21% oxygen. Therefore, changes in protein abundances during aestivation in normoxia would be due to the effect of aestivation alone. On the other hand, there could be a combined effect of aestivation and hypoxia on fish aestivating in hypoxia.

Overall, aestivation and hypoxia did not affect protein abundance of VEGF in heart which was constantly receiving oxygenated blood from the lungs. In the kidney, aestivation in normoxia increased protein abundance of VEGF during the maintenance phase (on day 12). Conversely, during aestivation in hypoxia, there were increases in the protein abundance of VEGF during both the induction and the maintenance phase. Structural and lectin-binding modifications in the renal corpuscle to allow the lungfish to cope with dehydration and suppression of urine excretion during aestivation account for the
increment. Aestivation in normoxia led to a decrease in the protein abundance of VEGF in the intestine as the lungfish no longer derive its energy from the assimilation of food. Instead, it taps on the internal stores of carbohydrates, amino acids, lipids and ketones for energy supply. By contrast, intestine of the lungfish aestivating in hypoxia showed no significant changes in the protein abundance of VEGF due to the low level of blood supply. Although aestivating in hypoxia resulted in no changes in the protein abundance of VEGF in the heart of the lungfish, hypoxia affects the protein abundance of Akt1 in this organ during maintenance phase, which was perhaps essential to the regulation of other molecules such as HIF to cope with the hypoxic conditions. In the kidney, aestivation in normoxia showed an increase of protein abundance of Akt1 in both induction and maintenance phases. During aestivation in normoxia, nitrogen metabolism involves the increase in urea production as the main form of excreted product (Loong et al. 2008b). Hence, by increasing the protein abundance of Akt1, other molecules could be up regulated to generate angiogenesis to elevate the efficiency of urea excretion in the kidney. On the other hand, nitrogen metabolism during aestivation in hypoxia involves both the suppression of ammonia and urea production (Loong et al. 2008b), as urea production would be too energy exhaustive to the aestivating lungfish. As a result, hypoxia induced a reduction of protein abundance of Akt1 to down regulate other angiogenic factors in collaboration with a decrease in nitrogen waste excretion through the kidney. Incidentally, in intestine, changes in the protein abundance of Akt1 is similar to that of VEGF i.e. there were no significant changes to the protein abundance of Akt1 during aestivation in hypoxia while aestivation in normoxia showed a decrease in the protein abundance of VEGF.

According to Thurston and Kitajewski (2008), the VEGF system has been conserved from zebrafish to man as a signaling pathway that is essential and rather specific to the vascular and haematopoietic systems. This statement is true for *P. annectens* as alignment of the partial nucleotide sequences of the coding region of the heart, kidney and intestine of the fish showed significant similarities. Therefore, it can be speculated that VEGF is highly conserved in the tissues of *p. annectens* and there should not be any tissue-specific VEGF isomers present in the different tissues of the lungfish. In addition, the phylogenetic tree constructed showed that VEGF is highly conserved in among the five species as bootstrap values of individual branches are more than 50. Lungfishes are well-associated with the evolution of water-land transition and they are of great interest because of their probable relationship as the sister group to tetrapods (Tohyama et al. 2000). This relationship is substantiated in the phylogenetic tree as nucleotide sequence of VEGF in the tissues of *P. annectens* is more clustered with the nucleotide sequence of VEGF in *Xenopus laevis* than in the fish. This implies that VEGF is a highly conserved signaling molecule that retained its nucleotide sequences in water-land transition.

REFERENCES


