Cross-linking of structural proteins in ageing skin: an in situ assay for the detection of amine oxidase activity

Abigail K. Langton · Christopher E. M. Griffiths · Michael J. Sherratt · Rachel E. B. Watson

Received: 5 March 2012 / Accepted: 16 August 2012 © Springer Science+Business Media B.V. 2012

Abstract With increasing age, dynamic tissues such as lungs, blood vessels and skin lose their ability to both deform and recoil, culminating in tissue stiffening. This loss of tissue elasticity, which profoundly impacts tissue function and thus morbidity, may be due not only to changes in the relative abundance of key extracellular matrix proteins within tissues but also to their accumulation of post-translational modifications. Whilst to date attention has focussed primarily on the age-related non-enzymatic formation of advanced glycation end products, the accumulation of pathological enzyme-mediated cross-links may also lead to age-related tissue stiffening. The lysyl oxidase (LOX) family of enzymes are constitutively expressed in adult tissues and are known to drive the catalysis of cross-links in both fibrillar collagens and elastin. Although immunochemical approaches are commonly used to localise the inactive pro-enzyme of LOX, and biochemical methods are employed to quantify activity in homogenised tissue, they do not allow for the in situ localisation of the enzyme. Thus, we have developed a novel assay to both detect and localise LOX enzyme activity in situ. LOX family members are amine oxidases and this assay uses the principle that an amine substrate in the presence of this class of enzyme will be oxidised to an aldehyde and hydrogen peroxide (H$_2$O$_2$). In turn, H$_2$O$_2$, when combined with luminol and horseradish peroxidase, will produce a light-emitting reaction that can be detected by film autoradiography. The development of a technique to localise specific amine oxidase activity in tissue sections may provide crucial additional information on the exact role played by this class of enzymes in mediating age-related tissue stiffening.

Keywords Amine oxidase · Cross-linking · Lysyl oxidase · Lysyl oxidase-like

Abbreviations

AGE Advanced glycation end products
BAPN β-Aminopropionitrile
DAPI 4',6-Diamidino-2 phenylindole
DEJ Dermal–epidermal junction
ECM Extracellular matrix
H$_2$O$_2$ Hydrogen peroxide
LOX Lysyl oxidase
LOXL Lysyl oxidase-like

Introduction

With advancing age, cutaneous (Escoffier et al. 1989), pulmonary (Lai-Fook and Hyatt 2000) and vascular (Mitchell 2008) tissues lose both tensile strength and compliance. This loss of tissue function can have a
profound impact on human morbidity. Age-related stiffening of these tissues is mediated primarily by remodelling of the collagenous and elastic fibre matrices (Bailey 2001; Robert and Labat-Robert 2000), the major protein components of which are the fibrillar collagens and elastin respectively (Kielty et al. 2002; Birk and Bruckner 2005). Characterisation of the molecular mechanisms which underlie age-related changes in these proteins is a vital first step in the development of preventative or reparative interventions (Sherratt 2009).

Elastic fibres are major components of the extracellular matrix (ECM) and are found in dynamic tissues such as the dermal compartment of the skin (Braverman and Fonferko 1982), lungs (Pierce and Hocott 1960) and blood vessels (Davis 1993). These fibres are composed of elastin and fibrillin-rich microfibrils and confer tissues with the ability to deform and recoil (Kielty et al. 2002). In contrast to elastic fibres, the fibrillar collagens, namely collagens types-I, -III, -V, -XI, -XXIV and -XXVII confer tensile strength to tissues by forming extended, mechanically stiff fibrils (Birk and Bruckner 2005). Fibrillar collagens are abundant in tissues including the dermis, tendon and cartilage. During development and maturation of dynamic tissues, the structural integrity of fibrillar collagens and elastic fibres rely heavily on the precise enzyme-driven formation of cross-links by the lysyl oxidase (LOX) family of enzymes (Csiszar 2001). Both collagen and elastin are stabilised by these enzymatic cross-links; however in elastin the function of these cross-links is to restrain excessive stretching, whilst in collagen the cross-links act by rendering the fibrils virtually inextensible (Bailey 2001).

Both elastic fibres and fibrillar collagens are uniquely long-lived (Sivan et al. 2008; Shapiro et al. 1991) compared to intracellular proteins (Jennissen 1995). The low turnover rate of these structural ECM proteins exposes them to degradation by a range of processes; enzymatic, chemical or biophysical. The long-lived nature of these proteins also makes them susceptible to the accumulation of age-related damage (Sherratt 2009). The accumulation of damage by biological molecules has long been recognised as a potential contributing factor to age-related functional decline (Partridge and Gems 2002; Vijg and Campisi 2008; Bailey 2001). Decline of physiological function may also result from the accumulation of post-translational modifications on and between structural ECM proteins, such that calcification (Winlove et al. 1996), lipiddation (Slatter et al. 2000) and aspartic acid racemisation (Clarke 1987) may occur.

One important modification that may compromise the structure, and hence function, of both collagen and elastin during ageing is the formation of pathological intermolecular cross-links. The uncontrolled accumulation of glucose and glucose-metabolite-derived cross-links are a common feature of many ageing tissues (Bailey 2001; Verzijl et al. 2000). These non-enzymatic cross-links undergo sequential modifications that culminate in the formation of advanced glycation end products (AGEs) (Paul and Bailey 1996). Whilst the non-enzymatic formation of AGEs is a well recognised causative mechanism of age-related tissue stiffening (Sherratt 2009), the role of the LOX family of enzymes as mediators of pathological enzyme-mediated cross-links has been largely overlooked. The LOX family of enzymes are copper-dependent amine oxidases (Csizsar 2001), which consists of LOX and four fully functional, but genetically distinct, lysyl oxidase-like proteins, LOXL, LOXL2, LOXL3 and LOXL4 (Molnar et al. 2003). LOXL has high sequence homology to LOX (Kenyon et al. 1993) and both enzymes catalyze the final step of collagen and elastin cross-linking (Coral et al. 2008; Trackman et al. 1992). LOX and LOXL are secreted as glycosylated pro-enzymes, that are proteolytically processed by procollagen C proteinase (bone morphogenic protein-1) into mature, biologically active forms (Trackman et al. 1992). Localisation of LOX family members has traditionally been performed using immunohistochemistry. However, these types of approach identify both the active and inactive form of the enzyme (Langton et al. 2011). Alternative biochemical methods can quantify the biologically active forms of LOX and LOXL in homogenised tissue but give no information regarding their localisation (Coral et al. 2008).

The ability to precisely localise the activity of the enzymes that drive cross-linking of elastin and fibrillar collagens may provide crucial additional information on the exact role played by LOX family enzymes in mediating age-related tissue stiffening. Therefore, we have developed a novel in situ assay that utilises the amine oxidase activity of LOX and LOXL to precisely localise them in unfixed tissue cryosections.
Experimental procedures

Reagents and tissue sample preparation

All reagents were obtained from the Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise stated. Human skin biopsy samples were obtained from the photoprotected buttock of young (age range 18–29 years; \( n = 9 \)) and aged (age range 65–75 years; \( n = 9 \)) healthy Caucasian volunteers. Local ethical committee approval (REC Ref. 09/H1006/23) was obtained for the study and all subjects gave written, informed consent. Skin biopsies were embedded in optimal cutting temperature compound (Miles Laboratories, Elkhart, IN, USA), snap frozen in liquid nitrogen and stored at \(-80^\circ C\). Samples were cryo-sectioned to a nominal thickness of 7 \( \mu m \) and mounted on SuperFrost Plus glass slides (Fisher Scientific UK Ltd, Loughborough, UK).

In situ activity assay

Cryosections were air dried at room temperature before use. Amine substrate solution was prepared by adding 1,4-diaminobutane to a final concentration of 40 mg/ml in phosphate buffer solution (5 mM di-sodium hydrogen phosphate, 5 mM sodium di-hydrogen phosphate monohydrate; pH 7.2). Horseradish peroxidase was added to a final concentration of 4 \( \mu l/ml \). Immediately prior to use, an equal volume of luminol (ECL chemiluminescence detection reagent 2; GE Healthcare, Buckinghamshire, UK) was added. Amine substrate solution containing luminol was applied to each tissue section and incubated at 37 \( ^\circ C \) for 5 min. Following incubation, excess amine substrate solution was carefully blotted from the tissue section and a glass coverslip was applied. The slides were placed in a developer cassette with an ECL film and incubated at 4 \( ^\circ C \) for 1 h. The film was fixed and developed using standard procedures. Inhibition of LOX and LOXL amine oxidase activity was performed by applying the LOX-specific inhibitor, \( \beta \)-aminopropionitrile (BAPN, 40 mg/ml phosphate buffer) to tissue sections for 5 min at room temperature. Following incubation, excess inhibitor solution was carefully blotted from the tissue section and the in situ activity assay was performed as described above. For comparison of enzyme intensity and distribution, samples from both young and aged individuals were performed in a single batch and detected on the same autoradiography film. Autoradiography films were scanned and the intensity of the signal produced was quantified using ImageJ software (Abramoff et al. 2004). Three line profiles (20 \( \mu m \) wide) per image were positioned at the dermal–epidermal junction (DEJ) and extended into both the epidermis and the dermis. Mean line profiles were calculated for all nine young and nine aged samples and profiles were compared using area under the curve analysis. Statistical analysis was performed using SPSS+ version 16.0 (SPSS Inc., Chicago, IL, USA).

Immunofluorescence staining

Frozen tissue sections (7 \( \mu m \)) were fixed in ice-cold acetone and hydrated in tris-buffered saline (TBS, 100 mmol l\(^{-1}\) Tris, 150 mmol l\(^{-1}\) NaCl; pH 7.4). Non-specific binding was blocked by incubation in 5 % normal rabbit serum. Mouse anti-human LOXL primary antibody (clone H-11, dilution 1:10; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) was applied for 1 h at room temperature. Sections were washed in TBS, prior to incubation with rabbit anti-mouse Alexa Fluor\(^{\circledR} 488 \) secondary antibody (Invitrogen; Paisley, UK). Nuclei were visualised by incubation of sections in 4',6-diamidino-2-phenylindole (DAPI). Images were captured using the All-in-one Type Fluorescence Microscope Biozero-8000 (Keyence; Osaka, Japan).

Results

Principle and methodology of the in situ amine oxidase activity assay

We have developed a novel assay to both localise and quantify relative amine oxidase activity in situ using tissue cryosections. The assay relies on the oxidation of an amine substrate in the presence of an amine oxidase to an aldehyde and hydrogen peroxide (H\(_2\)O\(_2\)). In turn, H\(_2\)O\(_2\) can be readily detected by the light-emitting horseradish peroxidase catalysed oxidation of luminol (Whitehead et al. 1979) (Fig. 1). The light output produced by the reaction is proportional to the amount of amine oxidation that has occurred. Substrate conversion and light output are constant as long as the substrate does not become limiting and the enzyme remains stable and active (Schwelberger and Feurle 2007).
The novel methodology developed here is straightforward to perform and can be completed in less than 2 h. Cryosections cut at a thickness of 7 μm were allowed to air dry, then a buffer solution containing the amine substrate, luminol (from a commercially available Western blotting detection kit) and horseradish peroxidase were applied for 5 min at room temperature. Excess solution was blotted from the tissue sections and glass coverslips were applied. Slides were exposed to autoradiography film at 4 °C for 1 h and then developed and fixed following standard procedures. The developed film produced a clear signal where chemiluminescence had been detected (Fig. 2; Table 1).

Application of the in situ amine oxidase activity assay in cutaneous tissue

In human skin, LOX and LOXL have previously been shown by immunohistochemical methods to have an overlapping distribution within the epidermis (Noble-sse et al. 2004). However, this type of analysis provides little information regarding the activity of the enzymes. Therefore, we have performed the in situ
assay on cryosections on biopsies from photoprotected human skin to localise enzymatic activity of these LOX family members. Enzymatic activity was localised to the epidermis, hair follicles, pilosebaceous glands and blood vessels of the skin (Fig. 3a, b). The same pattern of activity was consistently seen in tissue sections from different volunteer skin biopsies. Immunofluorescence staining was performed to localise LOXL protein (both inactive and active isoforms) within the skin (Fig. 3c) and tissue architecture was determined by the blue fluorescence staining of cellular DNA with DAPI (Fig. 3d). LOXL protein was localised to the identical compartments of the skin as those identified using the activity assay and was particularly intense in the epidermal basal layer (Fig. 3e). The widespread green fluorescence observed in the dermis is not LOXL immunoreactivity but it can be attributed to autofluorescence of the elastic tissue (Fig. 3c). The localisation of LOXL protein immunoreactivity within the epidermis, as opposed to the dermis, is in agreement with published data from other research groups (Noblesse et al. 2004).

In order to confirm the specificity of the in situ assay in detecting LOX/LOXL amine oxidase activity only, a control experiment was performed using the potent LOX/LOXL-specific inhibitor, BAPN. Skin cryosections were pre-treated with BAPN and subjected to the in situ activity assay. Following application of BAPN, we were unable to detect a chemiluminescence signal in the skin. Therefore, LOX/LOXL amine oxidase activity had been successfully inhibited and no alternative amine oxidases were detected within the skin (Fig. 4).

In situ detection of LOX/LOXL activity during cutaneous ageing

LOX/LOXL activity was also examined in young versus aged human skin using the in situ assay. In order to analyse the distribution of enzyme activity, the autoradiography films were scanned and quantified for the intensity of signal using ImageJ software. Line profiles extending from a midpoint at the DEJ to the outer surface of the skin and into the dermis were generated and analysed across the different age groups. Strong enzymatic activity was localised predominantly within the epidermis in both young and aged skin (Fig. 5a, b). However, the signal intensity was significantly increased in both the epidermis and dermis of aged skin when compared to young skin (area under the curve, arbitrary units; mean ± SEM; young: 10,353 ± 893; aged: 17,223 ± 1,118; *P* < 0.01, unpaired *t* test) (Fig. 5c). This finding reconciles with the previously published immunohistochemical detection of increased LOXL distribution in aged skin (Langton et al. 2011).

**Discussion**

Advantages of the assay and potential applications

The current study describes a novel assay that allows the localisation of amine oxidase activity in situ using unfixed tissue cryosections. The assay, which is rapid, reliable, highly sensitive and economical is based on the principle that an amine substrate in the presence of an amine oxidase will be oxidised to an aldehyde and H$_2$O$_2$ will be produced as a by-product (Whitehead et al. 1979).
The production of H2O2 during amine oxidation has previously been used to detect amine oxidase activity in a wide variety of tissue types, including ocular (Coral et al. 2008), placental (Kuivaniemi 1985), renal (Schwelberger and Feurle 2007) and murine skin (Hayashi et al. 2004) using both luminometric (Schwelberger and Feurle 2007) and fluorometric assays (Palamakumbura and Trackman 2002). However, whilst biochemical techniques can both rapidly and effectively detect and measure amine oxidase activity, the fact that they require homogenised tissue extracts dictates that these types of assay do not allow for the precise localisation of the enzyme activity within the tissue. Conversely, although immunohistochemical methods allow accurate localisation to be determined, this type of approach does not distinguish between the...

Fig. 3 The in situ activity assay was performed on tissue cryosections from photoprotected human skin. Enzymatic activity was localised to the epidermis, hair follicles, blood vessels and glandular tissue (a, b). Cryosections from photoprotected human skin were immunofluorescently labelled using a primary monoclonal antibody against LOXL (c) and tissue architecture was determined using DAPI (d). LOXL protein (both inactive and active isoforms) was localised to the epidermal basal layer of the skin (e). *Scale bar = 100 μm*

Fig. 4 The in situ activity assay was performed on skin cryosections from photoprotected human skin in the presence of the specific LOX/LOXL inhibitor BAPN. Amine oxidase activity was detected in the absence of inhibitor (a) however; pre-treatment of skin cryosections with inhibitor yielded no detectable signal (b). *Dotted line indicates the DEJ*
inactive and active forms of the enzyme. Therefore, we have developed this assay in order to both detect and localise only the active form of the amine oxidase enzymes.

This in situ activity assay is applicable to virtually any amine oxidase however, the decision regarding which amine substrate is suitable to use for a specific tissue type must be determined by the end user. To date, 33 human amine oxidase enzymes have been identified on the Universal Protein Resource database (UniProt 2011). These structurally heterogeneous enzymes can be subdivided into two main classes on the basis of the chemical nature of the associated co-factors. Flavine adenine dinucleotide is the co-factor of monoamine oxidases A and B and of an intracellular form of polyamine oxidase. A second group of amine oxidases contain topaquinone (Dove et al. 1996), a modified tyrosine side chain, utilized as a redox co-factor. This latter subfamily of amine oxidases is comprised of diamine oxidase, monoamine metabolizing semi-carbazide-sensitive amine oxidase and members of the LOX family (Kagan and Trackman 1991). In the current study we have examined cutaneous tissue which has no reported monoamine oxidase activity (Blaschko and Hellmann 1953; Hellmann 1955) and the only diamine oxidases known to be present are those of the LOX family (Csizsar 2001). LOX/LOXL require alkyl diamine substrates to be present for activity to be detected (Tang et al. 1989) therefore, we decided that 1,4-diaminobutane would be a suitable substrate for cutaneous tissue. Furthermore, following exposure to BAPN, the specific inhibitor of LOX/LOXL, we found no detectable enzyme activity. Therefore, we can conclude that in skin, using 1,4-diaminobutane as the substrate, this assay is specific for detecting LOX/LOXL activity.

Although we have used skin in this study, LOX and LOXL enzymes are critically required for the biosynthesis of functional ECMs in other tissues such as lung, aorta and ocular tissue (Csiszár 2001; Kuivaniemi 1985). In addition, LOX and LOXL are also implicated in many disease pathologies. Fibrosis is caused by the excessive accumulation of insoluble collagen fibres and increased expression of LOX and LOXL are implicated in several fibrotic diseases of the heart, mouth and kidney (Kagan 2000; Trivedy et al. 1999; Lopez et al. 2010). Emerging evidence also implicates LOX in promoting cell proliferation and metastasis of colorectal (Baker et al. 2011) and oral cancer (Trivedy et al. 1999). Thus, this in situ amine oxidase assay using 1,4-diaminobutane as the amine substrate, could be utilised in other tissues to precisely detect and localise enzymatic activity.

In this study we detected high LOX/LOXL activity in skin. This finding is consistent with previous reports where it has been shown that it contains a higher amount of LOX activity per mg of homogenised tissue than vasculature, fascia, pleura, lung, placenta and
cartilage (Kuivaniemi 1985). Although LOX and LOXL drive the formation of lysine-derived cross-links to provide optimum function to the tissue, when they are present in excess they may over-stiffen fibrillar collagens and elastic fibres (Bailey 2001). Therefore, using cryosections of aged skin we were able to precisely localise the activity of the enzymes that drive cross-linking of elastin and collagen. We found that activity was significantly increased in both the epidermis and dermis of aged skin compared to young skin. This finding indicates that LOX/LOXL may contribute to the accumulation of pathological cross-links and may play a role in mediating age-related tissue stiffening. However, it is unclear how increased epidermal amine oxidase activity can correlate with increased cross-linking of dermal fibrillar collagens and elastin. We hypothesise that amine oxidases may be produced by epidermal keratinocytes and that diffusion of these enzymes from the epidermis into the upper region of the dermis will be sufficient, at least in part, in facilitating the cross-linking of superficial dermal proteins. Alternatively, it may be that this class of enzyme has a previously unappreciated role within the epidermis. Further work is therefore required to elucidate the role of epidermal amine oxidase activity.

In conclusion, this assay is a useful tool that can specifically and precisely localise amine oxidase activity in tissue cryosections and could facilitate progress in the study of different aspects of amine oxidase regulation and biochemistry in a number of age-related pathological conditions.

Acknowledgments This work was supported by a programme grant from Alliance Boots, Nottingham, UK and by a Senior Age UK Fellowship awarded to MJS. CEMG is supported in part by the NIHR Manchester Biomedical Research Centre.

References

Blaschko H, Hellmann K (1953) Pigment formation from tryptamine and 5-hydroxytryptamine in tissues; a contribution to the histochemistry of amine oxidase. J Physiol 122(2):419–427